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T A B L E O F C O N T E N T S

ULTRAWEAK LUMINESCENCE ACCOMPANYING BIOCHEMICAL REACTIONS

Yu. A. Vladimirov

Although the existence of ultraweak luminescence in chemical and biological systems has been known for a long time, it was not until recently that the number of reports in this field increased tremendously. One possible reason for this snowballing of interest is the development of new methods for measuring the exceedingly weak radiation produced by chemical and biological systems; another, undoubtedly, is the increasing tendency to study biophysical and chemical phenomena at the submolecular (electron) level.

The concept of ultraweak luminescence takes in a very broad range of phenomena, and one may at first wonder whether the various forms of ultraweak luminescence actually have anything in common. In the present work, an attempt is made to demonstrate that although the reactions leading to the formation of products participating in the chemiluminescence reaction are different from system to system, the final stages culminating in the emission of a quantum of electromagnetic radiation have many features in common. The manifestation of chemiluminescence indicates that in the course of the process in question, excited molecules are formed; and the spectrum and kinetic behavior of the emitted radiation make possible the clarification of the role of excited states in the process or in the secondary reactions accompanying it.

A comparison of the phenomena of ultraweak luminescence in many different fields was facilitated by a photon-counter system including a photomultiplier cooled by liquid nitrogen.

FOREWORD

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During 1958-1959 at Moscow State University, Biophysics Department, F. F. Litvin and the author of this book began an investigation of ultraweak luminescence in biological systems using a photomultiplier cooled by liquid nitrogen and operating in a photon-counter system. The high response and relative simplicity of the apparatus, and especially the stability which makes it possible to obtain reproducible results, caused this method to come into use quickly in the studies of a number of other authors. The term "ultraweak luminescence" which we proposed came into wide use. This term, in a number of variants (ultrafaint luminescence, ultraweak chemiluminescence, etc.) is now used by most researchers working in this field. Meanwhile, it has recently become entirely obvious that this concept takes in an extremely broad range of phenomena, beginning with luminescence during reverse photochemical reactions and even in-

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*Numbers in the margin indicate pagination in the foreign text.

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cluding emission accompanying dark biochemical processes in tissues, a culture of yeast cells or a suspension of mitochondria. The question therefore may arise as to what extent it is feasible to consider all these seemingly completely different phenomena in a single book and whether in general there exists anything common to all forms of ultraweak luminescence other than the need for a particularly sensitive method for their detection.

In this book we will endeavor to demonstrate that although biochemical or photochemical reactions leading to the formation of products entering into the chemiluminescence reaction are not the same in different systems, the final stages of the process, ending with the luminescence of a quantum, have many features in common and only their comparison in a study of different systems makes possible an understanding of many very important characteristics of the luminescence mechanism. In accordance with this idea the author has striven /4 not only to demonstrate the widespread occurrence of ultraweak luminescence in biological systems, but also most importantly to consider the biochemical reaction mechanisms facilitating the process and the actual transformation of the chemical reaction energy into molecular excitational energy; in other words, we have attempted to approach the problem of ultraweak luminescence from the biochemical and biophysical points of view.

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Simultaneously with our first investigations on ultraweak luminescence in biological systems [32], R. F. Vasil'yev, V. Ya. Shlyapintokh and their associates began a systematic study of chemiluminescence accompanying oxidation and some other reactions in solutions of aromatic hydrocarbons and their derivatives [5, 11-25, 71, 72, 131, 189, 205-207]. Soon thereafter, Stauff and his colleagues [190-196] attempted to explain the mechanism for weak chemiluminescence during oxidation reactions of inorganic compounds. In 1961 a wide range of investigations on the ultraweak luminescence of tissues and homogenates and luminescence accompanying the oxidation of fatty acids was begun by B. N. Tarusov, A. I. Zhuravlev, A. I. Polivoda and associates [57-70, 98-101, 117-120]. The study of luminescence accompanying oxidation reactions of lipids and reactions involving peroxidase was begun independently by Anhström, Nilsson and associates [132, 182]. Almost simultaneously a number of authors discovered and investigated the luminescence of rat liver homogenates [37, 119] and yeast cell suspensions [37, 82, 191] and luminescence accompanying the enzymatic decomposition of hydrogen peroxide [37, 38, 74] and the oxidation of lipids [118, 119, 142]. Recently the number of publications devoted to this problem has increased sharply. Whereas after our study in 1959 [32] two years elapsed before the appearance of further reports on ultraweak luminescence in biological systems [118-120], the number of articles relating to this field (almost all by Soviet researchers) now exceeds a hundred*.

At first glance it might appear that the beginning of research on ultra- /6 weak luminescence was based on some discovery, and it would even be possible to dispute who made this discovery. However, it is sufficient to turn to history and learn that the first observations of ultraweak luminescence in biological systems and modern investigations on this problem were separated by a considerable time lapse. The fact that living tissue is capable of radiation in the optical region of the electromagnetic spectrum was postulated for the first time by A. G. Gurvich as early as the 1920's. Extensive literature was devoted to the study of the "mitogenic rays" which he discovered, but at present most investigators have departed from this problem and all these studies are practically forgotten. Individual researchers later repeatedly turned to the discovery of ultraviolet radiation from tissues, using Geiger-Müller counters as radiation detectors [82, 97, 103, 122, 135-137]. In 1954-1955 interesting observations of chemiluminescence from sprouts in the visible region of the spectrum were made by Colli, Facchini and associates [144-146] using a photomultiplier cooled with dry ice. However, not one of these studies was fated to become the beginning of a "chain reaction" of investigations of ultraweak luminescence. Why do we now observe such vigorous development in this field?

The reason apparently is to be found in the confluence of a whole series of circumstances. Undoubtedly, the most important of these is the development

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*For the reader interested in the biological aspect of ultraweak luminescence of plant and animal tissues we recommend familiarization with the monograph by B. N. Tarusov, I. I. Ivanov and Yu. M. Petrusevich entitled Sverkhslabyye Svecheniya Biologicheskikh Sistem. (Ultraweak Luminescence of Biological Systems.) Published by the "Vysshaya Shkola" publishing house.

of methods for recording the exceedingly faint radiation from biological objects.

On the other hand, the desire to use chemiluminescence to study the kinetics and mechanisms of processes was a stimulus for the investigation of ultraweak luminescence in chemical systems [18, 20, 72, 208] and in biochemical reactions; for example, in reactions involving peroxidase [155, 157, 182]. In particular, the presence of luminescence, as we will see later, indicates the participation of free radicals in the process. Measurement of ultraweak luminescence occurring during the recombination of unstable radicals apparently can supplement the electron paramagnetic resonance method, which will make it possible to detect and identify radicals stable in a particular system.

However, the main reason for interest in ultraweak luminescence was the /7 general trend in modern biophysics to study phenomena not only at the molecular level, but also at the submolecular, electron level. The idea of the need for investigation of submolecular mechanisms of biochemical processes has been expressed by many authors [30, 102, 115, 202, 203], but actual progress became possible only as a result of the development of new methods. Study of free radicals was raised to a new level after introduction of the electron paramagnetic resonance method: measurements of luminescence made possible clarification of the probability of electron transitions in molecules and the processes of energy transfer between molecules; investigation of the primary mechanisms of photoreactions shed light on the interrelationship between electron transitions and chemical reactions, etc. Chemiluminescence occupies a very definite place in this series of phenomena: its presence indicates that during the course of the process in question excited molecules are formed; and its spectrum and kinetic behavior make possible the clarification of the role of excited states in this process or in the secondary reactions accompanying it.

Thus, ultraweak luminescence may be regarded as one of the methods for studying the molecular energetics of biochemical reactions.

Although investigators always make some premises (frequently intuitive) as a point of departure when proceeding to work in a new field, the real importance of such a direction usually becomes apparent only at a time when everything has already been essentially clarified and the real investigation in this field is already being completed. The problem of ultraweak luminescence is far from such a stage. Here we can see only the first results, and the importance of the observed facts for most cases still cannot be fully evaluated.

In conclusion, a few words about terminology. The luminescence accompanying the chemical process, as is well known, is called chemiluminescence. If it is desirable to emphasize that reference is to a biochemical reaction, it is possible to use the word biochemiluminescence [38]. For luminescence accompanying reverse photochemical reactions the term photochemiluminescence was proposed in [38]. The term bioluminescence usually refers to the luminescence charac- /8 teristic of some bacteria, marine invertebrates, fungi, fireflies, etc., visible to the eye. This specific luminescence is found only in individual representatives of the animal and vegetable world, whereas ultraweak luminescence apparently occurs universally. We feel that the difference in intensity,

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mechanism, occurrence and biological role of these two types of luminescence makes it undesirable to accept the proposal by some authors [63] that these phenomena be combined under the common term bioluminescence. The interrelationships between different types of luminescence accompanying biological processes can be clarified using the following scheme:

Luminescence in biological systems

↙ ↘

Bioluminescence   Ultraweak luminescence

↙ ↘

Biochemiluminescence   Photochemiluminescence
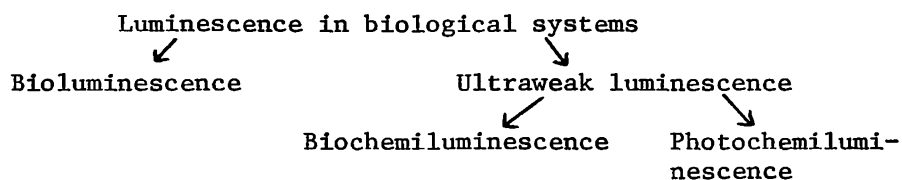
Mitogenic radiation was discovered by A. G. Gurvich in 1924 as a result of analyzing the mitotic distribution patterns in tissues. A. G. Gurvich concluded that the mutual influence of dividing cells on one another is exerted by means of radiation. The classical experiment of Gurvich involved measuring the number of mitoses in two connivent onion rootlets between which a quartz plate had been placed. In the experiment an increased number of mitoses was observed in comparison with an unirradiated rootlet. Just on the basis of this experiment A. G. Gurvich drew two conclusions basic for the entire "science of mitogenesis".

1. Under certain conditions living cells emit ultraviolet rays.

2. This radiation (also under certain conditions) can increase the number of mitoses in other cells; in other words, it is mitogenic.
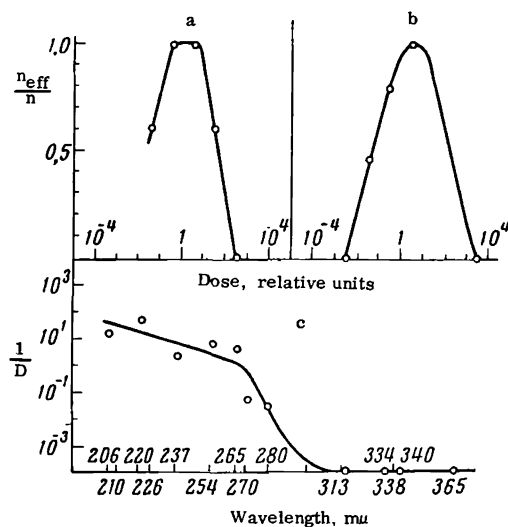

Figure 1. Dose Curves and Effect Spectrum of Mitogenic Radiation (Constructed using Data from [103]). a = Dependence of the Probability of Induction of Yeast Cell Budding on Dose at a Wavelength of 206 mµ; n_{eff} = Number of Experiments with a Positive Mitogenic Effect; n = Total Number of Experiments; b = Same as a, but for 220 mµ; c = Effect Spectrum; d = The Dose Corresponding to the Maximum Mitogenic Effect.

In the later experiments of Gurvich, mitogenic radiation was usually registered using a biological detector: for the increase in the number of mitoses in the cornea of the eye or for the increase in the number of buds in a yeast culture. The yeast method came into the widest use.

The yeast detector of mitogenic radiation is characterized by two interrelated peculiarities: a fantastically high sensitivity and the complexity of the dependence of the effect on the dose. According to estimates of the mitogenic effect of ultraviolet rays from physical sources, it is sufficient for a yeast cell to absorb one or even less than one effective quantum to cause formation of a bud [52]. This result can be explained only on the /10 assumption that absorption of a quantum by a particular cell causes secondary emission of many quanta as a result of some branching chain reaction. The nature of such reactions in the living cell was not clarified in the studies of Gurvich and his associates.

In ordinary photobiological processes an increase in the intensity of the effective radiation augments its effect. With an increase in the mitogenic radiation dose, stimulation of budding in yeast is replaced by its suppression (Figure 1a and b). This made it extraordinarily difficult

to use the yeast detector for quantitative investigations because in each case it was necessary to select the dose at which the budding effect was observed; the only answer was "yes" or "no". The effects themselves usually were small /11 (20-30% greater than the background) and the counting of the buds, in which the counter made a rather subjective choice ("still no bud", "bud" or "no longer a bud") not only required great skill, but also could not prevent systematic errors. Finally, not every yeast culture was suitable as a detector. In young, rapidly growing cultures the mitogenic radiation of the cells themselves was so high that additional irradiation no longer gave an effect. Expressed more briefly, a great many precautions were required in order to obtain a clearly expressed mitogenic effect, and there is nothing surprising in the simple fact that even the existence of mitogenic radiation was not confirmed in a number of laboratories. The book by Gurvich [54] gives a detailed analysis of the errors creeping into some of these studies; nevertheless he did not succeed in overcoming universal skepticism toward the "science of mitogenesis".

One of the principal reasons for the distrust of researchers toward the existence of mitogenic rays undoubtedly was that many of the facts obtained in the laboratory by A. G. Gurvich by mitogenic methods did not and still do not have a satisfactory scientific explanation.

For example, it was difficult to conceive how it was possible to measure the spectra of mitogenic radiation if the intensity of the light flux outside the monochromator slit was 0.1-1 quantum/cm^2·sec [38, 54]. It is not understandable why at the time of electron transitions the bands of individual radicals remain in the emission spectrum [38, 54] although it is known that photo- and chemiluminescence spectra are always characteristic for a molecule as a whole. It is difficult to visualize the mechanism of secondary emission from fresh solutions of glucose or glycocoll irradiated by mitogenic rays. The concept of enzymoids appears strange -- that is, the concept of high molecular wt. compounds which, having the corresponding enzyme as a nucleus, and any of the amino acids as the only substrate, possess enzymatic activity and are capable of unlimited "multiplication" during reinoculations [52, 86]. Ideas on the transformation of proteins from keto to enol forms under the influence of mitogenic radiation, and on the influence of this transformation on the stability of protein complex- /12 es and on the biosynthesis of protein are extremely naive.

Since A. G. Gurvich heatedly insisted not only on the authenticity of all these facts, but also regarded them as an indispensable part of a unified "science of mitogenesis", it is not surprising that the skeptical attitude toward individual, obviously erroneous results was extended to all the data obtained by mitogenic methods. We will see later that such an attitude was unjustified.

One of the leading problems arising in a study of luminescence associated with biochemical reactions is the problem of the reaction mechanisms, and in particular, the source of those relatively large portions of energy which were concentrated in light quanta. Mitogenic radiation falls in the ultraviolet region of the spectrum at wavelengths shorter than 270 mμ: radiation of longer wavelengths usually has no effect on mitoses (see Figure 1c). Since the portion of energy released during most enzymatic processes does not exceed 10 Cal/mole

for a single event (the energy of hydrolysis of ATP), the question naturally arises how such a large portion of energy as 100-150 Cal/mole (the energy of a quantum of ultraviolet radiation) can be released at once in the form of a quantum. In order to explain the origin of mitogenic radiation, Frankenburger postulated that quanta luminescence arises as a result of recombinations of free radicals forming as by-products in chemical and biochemical reactions [160].

Gurvich cites a detailed computation for the energy used during formation and released during the recombination of free radicals. It follows that luminescence in actuality can arise during the recombination of some of these radicals. In his schemes [50, 51, 54], which we do not cite here, because we feel that they have too formal a character, the essential role is played by oxygen, whose interaction with radicals yields additional energy for quantum luminescence. In actuality, it was discovered during the study of mitogenic rays that in all cases the presence of oxygen was a necessary condition for the appearance of luminescence [52, 54]. In some cases, according to computations which have /13 been made, the energy nevertheless does not suffice and its source could be visible light; the need for visible light illumination for the formation of mitogenic, that is, ultraviolet radiation, in actuality was discovered in a number of experiments [54].

Following initial familiarization with the studies of Gurvich one gets the impression that the deductive method was basic for this investigator; it is necessary to explain the existence of mitogenic rays and advance a speculative scheme requiring the participation of light and oxygen -- experimentation confirms this hypothesis. In actuality, this impression arises from the mode of exposition used by A. G. Gurvich in his studies. In actuality, the two main facts characterizing the development of mitogenic radiation, the need for oxygen, and in some cases a need for visible light as well, were discovered purely experimentally [52]. Thus, using the mitogenic method it was possible to discover empirically three basic phenomena exceptionally important for the problem which we are considering and subsequently confirmed by many methods using different objects.

1. The existence of luminescence during oxidation reactions in chemical and biochemical systems.

2. The need for oxygen in all these cases.

3. The possibility of using visible light to form a quantum of shorter-wavelength radiation.

On the basis of these results the conclusion was drawn that luminescence is associated with the formation of free radicals in the course of secondary reactions accompanying a basic chemical or biochemical process. As we will see later, all these data in one way or another were confirmed during the subsequent study of chemiluminescence which was carried out by other investigators, using other methods and frequently using other objects. This forces us to regard the "science of mitogenesis" with less skepticism, at least that part of it applying to the formation of weak luminescence during chemical and biochemical processes.

Brightly luminescent compounds are the best material for an investigation of photoluminescence. The situation is completely different in a study of chemiluminescence. Strong chemiluminescence at room temperature is observed in the system: hydrogen peroxide + iron compound + luminol:

$$H_2O_2 + \text{(luminol structure)} \xrightarrow[\text{luminescence}]{Fe^{++}}$$
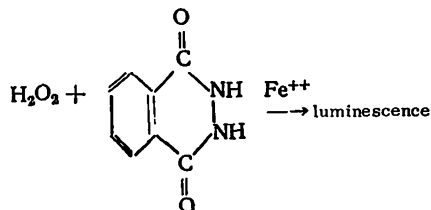
However, precisely this reaction, whose study has been carried out over the last 30 years [112], was so complex that its mechanism still remains far from being clarified [212, 213].

Many interesting results have been obtained during the study of another reaction -- the oxidation reaction of pyrogallol by hydrogen peroxide -- which also is accompanied by relatively strong luminescence and was observed visually by Trauts as early as 1905 (cited in [133]). However, in this case as well the reaction mechanism was not sufficiently clear. Here we will not discuss the other reactions also accompanying bright chemiluminescence [138, 185] because the mechanism of this luminescence has been poorly studied, in the first place, and in addition it apparently differs from the mechanism of ultraweak luminescence in biochemical systems.

The use of photon counters constructed using gas-discharge tubes or sensitive low-noise photomultipliers made it possible to detect weaker luminescence /15 in a great many oxidation reactions of inorganic and organic compounds. It is the mechanism of such very weak chemiluminescence which now has been most satisfactorily studied due to systematic investigations carried out, in particular, by Audubert in 1938-1939 [97, 135, 136] and recently by Stauff and his colleagues [190-196], as well as by R. F. Vasil'yev, V. Ya. Shlyapintokh, O. N. Karpukhin and others [11-25, 71, 72, 131, 189, 205-208]. Some of the results of these studies will be discussed here.

Chemiluminescence in Aqueous Solutions

Examples of the reactions accompanying luminescence in the ultraviolet and visible regions of the spectrum are shown in Tables 1 and 2, compiled using data from studies by Audubert [136], Stauff and Schmidkunz [193]. Almost all the reactions investigated by these authors were characterized by three peculiarities.

TABLE 1. REACTIONS ACCOMPANIED BY CHEMILUMINESCENCE
IN THE ULTRAVIOLET REGION OF THE SPECTRUM [136].

Reagents	Luminescence intensity, thousand quanta/$cm^2 \cdot$sec	
	at 200 mμ	at 240 mμ
NaOH + HNO_3 (or H_2SO_4)	2–3	7–10
K_2SO_3 + O_2	1.5–2	–
$Na_2S_2O_3$ + O_2	1–1.5	7–10
pyrogallate + O_2	1–1.5	10–15
C_2H_5OH + $HCrO_4$	–	70–100
$K_2C_2O_4$ + Br_2	–	10–15
$K_2C_2O_4$ + I_2	–	7–10
glucose + $KMnO_4$	1.5–2	–

1. In each elementary event of the main process there is release of considerably less energy than contained in a chemiluminescence quantum. For example, according to data given by Audubert, oxidation reactions during which 12–32 Cal/mole are released are accompanied by emission of luminescence in the <u>/16</u> region 240–260 mμ; the quanta energy of this radiation* is 120–130 Cal/mole [136].

2. In most cases the efficiency of the photon luminescence process is <u>/17</u> very small: for each molecule entering into the basic chemical process there is a release of 10^{-12}–10^{-15} quanta. At the same time, the quantum yield of chemiluminescence in the ultraviolet region ($1 \cdot 10^{-15}$–$1.4 \cdot 10^{-15}$ for the oxidation of propyl gallate or K_2SO_3 by molecular oxygen [136]) on the average was considerably lower than in the visible region of the spectrum (10^{-9} quantum/ radical for the oxidation of K_2SO_3 [195]). We will return to the cause of this phenomenon on pp. 40–43.

3. The presence of oxygen is necessary for chemiluminescence not only in those cases when oxygen is a participant in the basic process or when it may be released in the course of the reaction (peroxide decomposition and reactions involving hypochlorite), but also in such reactions as the neutralization of an alkali by an acid [193], where the basic process transpires without any participation of oxygen.

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*The relation between the energy of a quantum (E, Cal/mole) and wavelength (λ, mμ) is defined by the equation E = 28,600/λ.

TABLE 2. EXAMPLES OF REACTIONS ACCOMPANIED BY
WEAK LUMINESCENCE IN THE REGION 250-650 mμ [193]*.

Type of reaction	Reagents	Maximum luminescence intensity, relative units	Duration of luminescence, minutes	Remarks
Interaction with oxygen of the air (air passed through solution)	NaOH (1 M) + HCl (2 drops)	> 200	1-2	In the absence of oxygen the neutralization reaction is not accompanied by luminescence
	NaOH (1 M) + HCl (2 drops) + fluoresence	> 400	10	
	$NaHSO_3$ (0.001 M)	> 500	–	–
	benzyl Na	> 500	15-20	–
	NaClO	100	–	–
Interaction with H_2O_2 (2 ml of 30% solution of H_2O_2 + 55 ml solution of reagent)	NaClO	–	0.5	Visible dark red luminescence
	formaldehyde (15%) + NaOH (2 M)	> 300	7	–
	pyrogallol (10%) + NaOH (2 M)	> 100	7	–
	Fe^{++} + EDTA (catalyst)	> 200	0.3	–
	Fe^{++} + EDTA (catalyst) + gelatin (0.3%)	> 200	0.03-0.05	–
Oxidation using NaClO (1 ml NaClO + 55 ml solution of reagent)	urea (0.05 M)	200	2	–
	guanidine (0.05 M)	200	–	–
	dimethylsulfoxide (90%)	> 1000	10-12	Luminescence clearly visible to the eye

*[193] gives examples of many other reactions whose chemiluminescence intensity falls in the range from 2 to 200 relative units.

All these facts found their explanation in the circumstance that a quantum is not emitted during the course of the main chemical process, but as a result of improbable secondary reactions which lead to the formation of free radicals.

Study of the mechanism of the chemiluminescence processes usually includes: a) study of the total energy involved in the process, and in particular, an estimate of the energy released during recombination of the surmised radical participants of the reaction; b) investigation of the kinetics of all reactions possible in the particular system and their comparison with the kinetics of luminescence; and finally, c) measurement of the spectral composition of chemiluminescence and its yield in the absence and in the presence of activators.

In attempting to explain the obvious noncorrespondence between the energy of a chemiluminescence quantum (hν) and the heat of reaction (Q or ΔH), Audubert [136] postulated that the luminescent quantum also includes the activation energy (E) of the process:

$$h\nu = E + Q. \tag{1}$$

The basis for this, in particular, was that in a number of cases the luminescence activation energy was extremely high. However, even despite this, equation (1) was not observed and it was necessary to assume that the luminescence was caused by the recombination of two radicals, including E and Q   /18 [136]:

$$h\nu = 2E + 2Q. \tag{2}$$

The approximate satisfaction of equation (2) was demonstrated for a number of oxidation processes; however, the luminescence spectrum was not measured in a single case and it was only possible to estimate the mean energy of the quanta in the different experiments: 145-150, 122-145 or 122-155 Cal/mole [136].

It now is well known that all the reactions studied by Audubert also are characterized by luminescence in the visible region of the spectrum (400-600 mμ), that is, equation (1) also may be valid.

How and to what degree the thermal energy (activation energy) can be transformed into the light energy of photons will be considered in Section V, using several simple systems as examples. In studying the oxidation reactions of inorganic and organic compounds, most researchers assume that the formation of free radicals (accumulation of E) is thermally activated and the appearance of excited molecules occurs during the interaction of these radicals (ΔH ≈ hν). Such concepts lie at the basis of the Gurvich [52-54], Vasil'yev [11-13] and Stauff [193-195] methods. In this approach there is an immediate narrowing of the number of considered reactions, since even in the case of recombination of free radicals there is immediate release of 40-50 Cal/mole, that is, an energy equal to a quantum of visible light.

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In aqueous solutions the source of such energy may be the following radical recombination reactions:

1) $2\overset{\bullet}{O}H \rightarrow H_2O_2^*(\Delta H = 47 \text{ Cal/mole})$;

2) $2\overset{\bullet}{O}_2H \rightarrow H_2O_2^* + O_2$;

   or $2\overset{\bullet}{O}_2H \rightarrow H_2O_2 + O_2^*(\Delta H = 58 \text{ Cal/mole})$;

3) $\overset{\bullet}{O}_2H + \overset{\bullet}{O}H \rightarrow H_2O + O_2^*(\Delta H = 77 \text{ Cal/mole})$, etc.

It therefore may be assumed that a considerable part of the reactions considered in Tables 1 and 2 is related to the formation of these radicals. In particular, an appreciable luminescence accompanies the decomposition process of H_2O_2 (for example, in the presence of Fe^{++} and EDTA [193, 196]), where, as is /19 well known, these radicals are formed. The direct determination of free radicals during the course of the reaction of sulfite oxidation by oxygen in the presence of metals was accomplished by Stauff and his colleagues [195].

As indicated in the references, the closeness of luminescence spectra during oxidation and neutralization reactions also supports the fact that the recombination of water radicals actually leads to chemiluminescence in these reactions. Despite the difficulty of measuring the chemiluminescence spectra due to the weakness of luminescence, Stauff and Rümmler [192] carried out such measurements for three reactions. Comparing the position of the luminescence maxima with the position of the vibrational maxima in the absorption spectrum of the van der Waals oxygen complex, $(O_2)_2$, due to the $S \rightarrow T$ transition, Stauff and his associates [192, 194] concluded that this complex in an excited state is formed in oxidation reactions and is the direct "emitter" of chemiluminescence quanta (Table 3).

TABLE 3. POSITION OF ABSORPTION MAXIMA (λ_{abs}) OF $(O_2)_2$ COMPLEX AND CHEMILUMINESCENCE (λ_{chemi}) MAXIMA [194].

λ_{abs}	Absorption intensity, relative units	λ_{chemi}	Reaction
630	60	634.8	H_2O_2 + NaClO
578	75	580	urea + NaClO
533.5	25	535	urea + NaClO
477.5	50	480	NaOH + H_2SO_4 + O_2
381.5	30	360–390	NaOH + H_2SO_4 + O_2
345.3	10		

The authors feel that the energy aspects of the processes explain why in different reactions there is transition of an oxygen molecule to different vibrational sublevels. For example, in the recombination

$$\overset{\bullet}{O}H + \overset{\bullet}{O}H \rightarrow H_2O_2$$
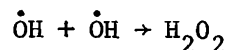
47 Cal/mole are released; this is only adequate for a transition to the lowest <u>/20</u> oxygen sublevel, which corresponds to absorption at 630 mμ*.

In the reaction

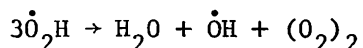$$\overset{\bullet}{O}_2H + \overset{\bullet}{O}_2H \rightarrow H_2O_2 + O_2$$
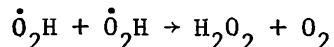
there is a release of 58 Cal/mole; this is adequate for transition to sublevels corresponding to 534.2 and 577.1 mμ.

A still greater energy (77 Cal/mole) is released during recombination of radicals

$$\overset{\bullet}{O}_2H + \overset{\bullet}{O}H \rightarrow H_2O + O_2;$$
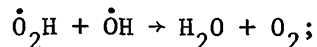
in this case there can be a transition to an excited level causing absorption at 380.5 mμ.

In the reaction

$$3\overset{\bullet}{O}_2H \rightarrow H_2O + \overset{\bullet}{O}H + (O_2)_2$$

there is release of 89 Cal/mole.

It is not entirely clear to what extent estimates of the chemiluminescence maxima may be precise and why the luminescence of a luminescence quantum in solution occurs in dependence on the excitation method from different vibrational sublevels of the van der Waals complex, and not from the lowest level, as occurs in photoluminescence and chemiluminescence during the oxidation of aromatic hydrocarbons. However, the Stauff hypothesis nevertheless satisfactorily explains the important role of peroxide radicals and water radicals and also molecular oxygen for chemiluminescence reactions. Measurements of the reaction kinetics of urea oxidation by sodium hypochlorite [193] and hyposulfite by molecular oxygen [194] are also in agreement with these ideas. The proposed models

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*The chemiluminescence bands at 633 and 703 mμ, corresponding to the $(O_2)_2$ vibrational sublevels, were observed when H_2O_2 was added to HClO. The presence of a well-defined vibrational structure in this case can be attributed to the fact that molecules of gaseous oxygen were excited at the surface of bubbles released during the course of the reaction [171].
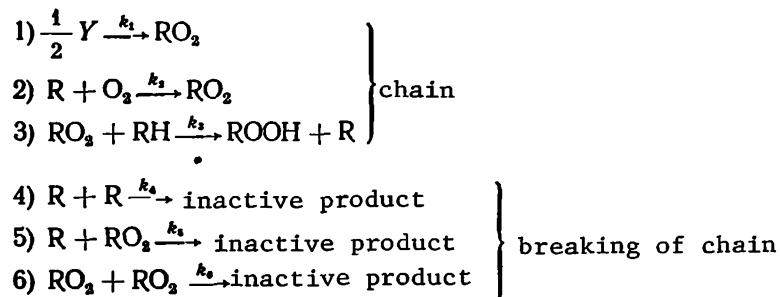
14

of these processes were considered in greater detail in the mentioned studies.

Chemiluminescence in Oxidation Reactions of Aromatic Hydrocarbons

Using a sensitive apparatus for measuring chemiluminescence, whose basis was an end-type photomultiplier cooled with dry ice [16, 23], V. Ya. Shlyapintokh, R. F. Vasil'yev, O. N. Karpukhin and their associates discovered luminescence accompanying a whole series of reactions, including thermal decomposition of organic hydroperoxides in solution, decomposition and oxidation of organic compounds in the gas phase, electrochemical reactions, solution, condensation and polycondensation [22, 189]. However, the most detailed study was made of luminescence during the chain oxidation reaction of aromatic hydrocarbons.

This investigation was facilitated from the very beginning by the fact that the mechanism of chain oxidation has been sufficiently studied by other methods. Here are its basic stages (cited in [18]):

$$1) \frac{1}{2}Y \xrightarrow{k_1} RO_2$$
$$2) R + O_2 \xrightarrow{k_2} RO_2$$
$$3) RO_2 + RH \xrightarrow{k_3} ROOH + R$$
chain

$$4) R + R \xrightarrow{k_4} \text{inactive product}$$
$$5) R + RO_2 \xrightarrow{k_5} \text{inactive product}$$
$$6) RO_2 + RO_2 \xrightarrow{k_6} \text{inactive product}$$
breaking of chain

Here Y is a molecule of the chain oxidation initiator; R is the hydrocarbon radical heading the chain; RO_2 is the peroxide radical; RH is the hydrocarbon; ROOH is the hydroperoxide. The total quantum yield of luminescence in this process is η_{chemi}, that is, the number of quanta emitted for each formed molecule of the peroxide does not exceed 10^{-8}. This means that only some of the reactions cited above are accompanied by luminescence. They obviously may be processes resulting in the release of much energy. Table 4 gives the values of the activation energy and heat of reaction of the chain oxidation of hydrocarbons taken from [11, 13].

TABLE 4. ACTIVATION ENERGY E_a AND HEAT ΔH OF REAC-
TION FOR HYDROCARBON CHAIN OXIDATION (IN KCAL/MOLE).

Number of reaction	Reaction	E_a	ΔH
2	$R + O_2 \rightarrow RO_2$	0	+24

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TABLE 4. (Continued)

Number of reaction	Reaction	E_a	ΔH
3	$RO_2 + RH \rightarrow ROOH + R$	15	+3
4	$R + R \rightarrow R_2$	0	+60 – +90
5	$R + RO_2 \rightarrow ROOR$	0	+70
6	$RO_2 + RO_2 \rightarrow O_2 +$ molecular products	0	+100

Table 4 shows that luminescence may accompany radical recombination reactions 4, 5 and 6. In this case oxygen plays a very important role in chemiluminescence. In the presence of oxygen in a concentration of only 10^{-6} M, reaction 2 transpired so rapidly that reactions 4 and 5 to all intents and purposes did not occur and all the luminescence apparently was caused by reaction 6 [18]; in the absence of oxygen, luminescence was also observed, obviously as a result of recombination of the radicals R + R (reaction 4), but it had a considerably lesser intensity [19]. This was caused primarily by the low yield of chemiluminescence in the latter case, which in turn may be related either to a low percentage of excited molecules of the product (η_p^{ex}) or to a low quantum yield of luminescence of this product (η_p) [5, 13]:

$$I = \eta_p \cdot \eta_p^{ex} \cdot \omega = \eta_{chemi} \cdot \omega, \qquad (3)$$

where I is the intensity of chemiluminescence and ω is the rate of reaction.

Investigation of the kinetics of the processes confirmed that it was in fact reactions 4, 5 and 6 which were responsible for the luminescence [11, 18, 22]. Later measurement of chemiluminescence was used for determining the rate constants for the liquid-phase oxidation reaction of hydrocarbons [11, 18] and for a study of biochemical processes.

Now we will discuss in greater detail the problem of identifying a molecule which is in an excited state as a result of a chemical process.

It is known that in the identification of molecules responsible for photo-/23 luminescence in a particular system án important role is played by measurement of the luminescence excitation spectra, luminescence spectra, quantum yields and duration of luminescence attenuation.

However, in the study of chemiluminescence, instead of measuring the excitation spectrum, in the optimal case it is the practice to compute the energy

of the reaction and to measure the luminescence spectra and yields, as well as the duration of the excited state of molecules; this is exceedingly complicated.

Nevertheless, Vasil'yev and his associates made such measurements. In the studies of these authors the evaluation of the duration of the excited state of a luminescent molecule was made using the coefficient of luminescence quenching value for high oxygen concentration. Under these conditions

$$\frac{I_0}{I} = \frac{\eta_0}{\eta} = 1 + k\tau_p[O_2], \tag{4}$$

where I_0 and η_0 are the intensity and yield of luminescence in the absence of a quencher (that is, at low oxygen concentrations; I and η are the same in the presence of a quencher; τ_p is the lifetime of the excited state of a molecule in the absence of a quencher. Plotting on a graph

$$\frac{I_0}{I} = f[O_2], \tag{5}$$

the authors determined the value of $k \cdot \tau_p$, which was equal to $2 \cdot 10^3$ ℓ/mole. Since the diffusion coefficient of oxygen is $\approx 10^{10}$ ℓ/mole·sec (for the quenching of aromatic hydrocarbon fluorescence) or $4 \cdot 10^9 - 5 \cdot 10^9$ ℓ/mole·sec (for the quenching of phosphorescence in these same compounds), the lifetime of an excited state of a molecule forming during chemiluminescence was found to equal 10^{-7} sec (if the product is in a singlet state) or 10^{-6} sec (if the product is in a triplet state) [13, 14, 24]. However, this value to a considerable degree is dependent on the process of intramolecular deactivation of the excited state of a molecule. The probability of such a process in molecules of a newly-forming product can be very high, which should be reflected by a decrease in the /24 quantum yield of luminescence from the product. It was estimated in the studies of Vasil'yev and Vichutinskiy [11, 20, 21] by activating chemiluminescence with luminophors. In this phenomenon the addition of a small quantity of luminescent activator (such as dibromoanthracene) to an oxidative hydrocarbon solution (such as cyclohexane or ethyl benzene) leads to an increase in the intensity of luminescence, change of the spectrum and suppression of the quenching effect of oxygen as a result of energy transfer of the excited state:

$$P* + A \rightarrow A* \rightarrow A + h\nu_{fl}^A$$
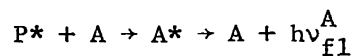
With an increase in the concentration of the activator the intensity of its luminescence tends to some limit which will be attained when all the excited molecules of the product P* transfer their energy to molecules of the activator (A). The ratio of the intensity of luminescence (I_A) in this case to the intensity of luminescence without an activator (I_p), with the sensitivity

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of the apparatus to the luminescence spectra taken into account, is equal to:

$$\frac{I_A}{I_p} = \frac{\eta_A}{\eta_p}, \qquad (6)$$

where η_A is the quantum yield of activator fluorescence; η_p is the quantum yield of product fluoresence. Knowing I_A/I_p and η_A (from data obtained by measuring photoluminescence) it is possible to find η_p, which in the considered case was equal to $10^{-4} - 10^{-3}$ [11, 13, 14, 21].

It is known that the natural lifetime of a molecule (in the absence of inter- and intramolecular quenching) is equal to [121]

$$\tau_0 = \frac{\tau}{\eta}; \qquad (7)$$

since $\tau = 10^{-7} - 10^{-6}$ sec, and $\eta = 10^{-4} - 10^{-3}$, then $\tau_0 = 12^{-2} - 10^{-3}$ sec. This means that in the chemiluminescence process molecules are formed in a triplet state whose natural lifetime ($10^{-2} - 10^{-3}$ sec) is decreased to 10^{-6} sec as a result of the strong deactivation of triply-excited molecules at room and high- /25 er temperatures ($\eta_p = 10^{-4} - 10^{-3}$).

No matter how complex the direct measurement of the chemiluminescence spectra, it was accomplished by Vasil'yev and Rusina [24] using a high-transmission monochromator with a relative aperture of 1:2 and with curved slits 7 cm in height [16]. Although the derived spectra fell in the near region (maxima 420-520 mμ) they were different for ethyl benzene, cyclohexane, n-decane and methyl-ethyl ketone. In all cases they corresponded to the luminescence spectra of ketones forming during the recombination of peroxide radicals [24]:

Peroxide radical of   Acetophenone   Phenyl Ethanol
ethyl benzene
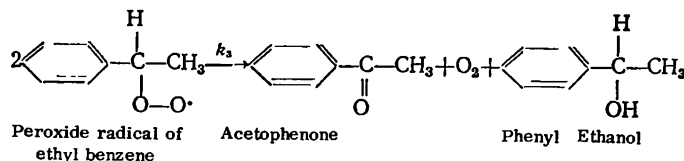
Particularly convincing is the coincidence of the chemiluminescence spectrum with the photoluminescence spectrum for forming diacetyl, as demonstrated in the oxidation of methylethyl ketone [24]:

Peroxide radical of   Diacetyl
methylethyl ketone

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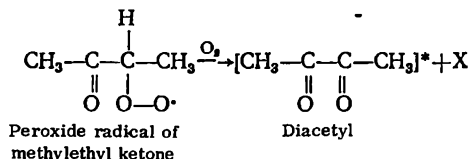
The excited state of ketones is a triplet characterized by a natural life-time of $10^{-2} - 10^{-3}$ sec, which corresponds to the lifetime of the products form-ing during chemiluminescence. The luminescence spectra of different ketones are extremely close. These data serve as additional arguments that it is the ketones and forming peroxide radicals during recombination (during the dispro-portionation reaction) which are in an excited state. However, these data do not exclude the possibility of simultaneous formation of excited oxygen mole-cules from van der Waals complexes $(O_2)_2$.

Thus, as a result of investigations of chemiluminescence accompanying the <u>/26</u> oxidation of aromatic hydrocarbons it was possible to obtain a detailed explana-tion of the principal empirical patterns of behavior characteristic in general for the processes of chemiluminescence in chemical and biochemical systems: a low yield of luminescence, and a need for oxygen and closeness of the spectra for the oxidation of quite different compounds. As a result of these studies it became clear that a low yield of luminescence $\eta_{chemi} = 10^{-8}$ was caused both by a low yield of luminescence of the forming excited ketones ($\eta_p = 10^{-4} - 10^{-3}$), and also by the fact that during the course of the reaction only an insignifi-cant percentage of the ketone molecules were excited (using formula (3) we find $\eta_p^{ex} = 10^{-4} - 10^{-6}$ [14]). The role of oxygen is in the transformation of radi-cals whose recombination leads to the formation of nonluminescent dimers RR into ROO$^\cdot$ radicals, whose recombination causes formation of luminescent ketones. The closeness of the spectra during the oxidation of different hydrocarbons is caused not by the fact that in all cases the same compound is formed (as postu-lated by Stauff for the oxidation reactions of simpler substances), but by the fact that in all cases ketones are formed whose luminescence spectra are caused by the carbonyl group and have little dependence on the structure of the remain-ing part of the molecule.

Unfortunately, the processes accompanying chemiluminescence in biological systems are characterized by a considerably greater complexity and until now there have been no investigations of the kinetics and mechanism of luminescence in biochemical reactions which in a careful and systematic approach come any-where near the investigations of chemiluminescence accompanying the oxidation of hydrocarbons.

As already mentioned, ultraweak ultraviolet radiation from biological objects was discovered for the first time by A. G. Gurvich using a biological detector [52, 53, 54, 165]. The most vulnerable spot in the study of these mitogenic rays was that the biological objects served both as the radiation source and its detector. The need for investigations of luminescence by objective methods was understood by many, and as early as the 1930's attempts were made to develop and use sensitive detectors for the recording of weak ultraviolet rays. The first successes were obtained in work with counters for ultraviolet photons -- modified Geiger counters with a quartz window and a metal photocathode. In particular, by use of this method S. R.Rodionov and G. M. Frank [103] discovered luminescence during the contraction of a muscle and at the time of stimulation of a nerve fiber, and Audubert investigated the luminescence accompanying not only a number of chemical reactions of the oxidation type, but a number of enzymatic reactions as well [135, 136]. Later Becher discovered the luminescence of fibroblasts [137] and N. A. Troitskiy, S. V. Konev and M. A. Katibnikov observed the ultraviolet radiation of a yeast culture [82, 122].

All these authors noted considerable difficulties in such investigations caused by the instability of the Geiger tubes operating as photon counters. The development of more modern counters (for example, see [127]) possibly changes the situation, but today it is not Geiger counters but photomultipliers with a small dark current (as a result of the design peculiarities or as a result of cooling by solid carbon dioxide or liquid nitrogen) which have recommended themselves as the most effective and promising instruments for measuring ultraweak /28 luminescence in biological systems.

In all probability Arnold and Strehler must be regarded as the pioneers in the investigation of the weak luminescence of biological objects. The apparatus developed by these authors consisted of a photomultiplier which was completely submerged in a Dewar vessel containing liquid nitrogen. Opposite the silvered window in the Dewar vessel there was a test tube containing the solution to be tested or the capillary of a flow-through system. The pulses arriving from the photomultiplier were registered by a counter [134, 198]. In other words, this apparatus in principle already contained all the basic elements of the apparatus used at the present time. This light detector was used in a study of the bioluminescence of a firefly and bacteria, and a prolonged post-luminescence from green leaves was discovered [198, 199]. The discovered "photosynthetic luminescence" was a relatively strong luminescence caused by inverse photochemical reactions in chloroplasts. Study of the luminescence arising in dark biochemical reactions (if the specific mechanisms of bioluminescence are not taken into account) was not made by Strehler, and such a problem apparently was not formulated.

In 1954 a group of Italian investigators developed a sensitive apparatus which incorporated an end-type photomultiplier cooled with solid carbon dioxide. In these studies it was found that there is a luminescence from sprouts of wheat, corn, lentils and beans in the visible region of the spectrum (450-650 mμ) which was observed without preliminary illumination of the object, that is, was caused by dark biochemical reactions in the sprouts [144, 145].

Study of ultraweak luminescence in biological systems for the purpose of checking the data of A. G. Gurvich (see Section I), on the one hand, and for clarifying the role of excited states during biochemical reactions, on the other, was undertaken by the author in collaboration with F. F. Litvin in 1959 with the development (on the basis of Soviet-produced instruments) of quite sensitive apparatus [32]. The apparatus consisted of a photomultiplier cooled by liquid nitrogen and a pulse counter (Figure 2); it made it possible to register each /29 tenth quantum incident on the photomultiplier photocathode and to detect a light flux of 100 quanta/min in the region 400-500 mμ or 500 quanta/min in the region 650-850 mμ. Instruments of such a type apparently are among the most sensitive light detectors known at the present time. A description of the apparatus and work with it is given in our book [35]. A number of papers describe the problems involved in using photomultipliers cooled with liquid nitrogen [32, 67, 98, 129] and solid carbon dioxide [11, 16, 23, 146, 193] and the use of low-noise uncooled photomultipliers [125, 164] for measuring weak luminescence in chemical and biological systems.
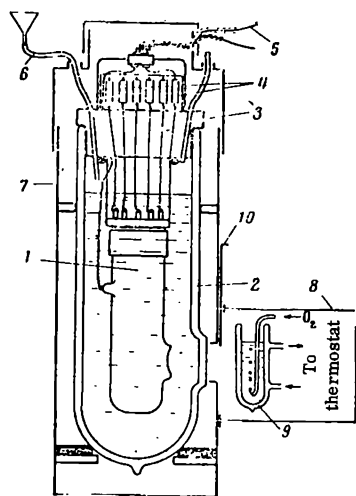

Figure 2. Diagram of Apparatus for Studying Ultraweak Luminescence [32, 35]. 1 = Photomultiplier; 2 = Dewar Vessel with Liquid Nitrogen; 3 = Foam Plastic Plug; 4 = Voltage Divider; 5 = High Voltage Input and Signal Output; 6 = Rubber Tube with Funnel for Liquid Nitrogen; 7 = Outer Metal Housing; 8 = Light-Proof Chamber for Object; 9 = Thermostat Regulated Vessel for Suspension to be Tested; 10 = Shutter and Light Filter.

Using the developed instrument we first confirmed the data obtained by Strehler on the prolonged (up to an hour or more) post-luminescence of leaves [198, 199] and on the chemiluminescence of riboflavin under the influence of hydrogen peroxide [200], and also discovered the post-luminescence from dry proteins [32]. Later the apparatus was used for a study of the low-temperature phosphorescence of proteins [33] and for a study of chemiluminescence in the course of dark processes accompanying photochemical reactions involving chlorophyll [84, 89]. We also succeeded in confirming the data obtained by Colli and his associates on the faint luminescence from bean sprouts. Later, in attempting to register the chemiluminescence from yeast cell suspensions in darkness, /30 we discovered a light-induced luminescence from yeasts which was closely associated with cell metabolism [37, 38].

The method which we developed for measuring ultraweak luminescence using a photomultiplier cooled with nitrogen came to be used in studies by other authors. In 1961 B. N. Tarusov, A. I. Zhuravlev and A. I. Polivoda discovered that in the tissue homogenates of irradiated rats there is a chemiluminescence at 37° and especially at 60-70°C, whereas homogenates from unirradiated animals had an insignificant luminescence only at 50° [118, 119]. A particularly interesting observation of these authors was that in a living rat with an opened abdominal cavity there was luminescence from the liver which was stronger than in irradiated animals [119, 120].

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Some results obtained in these investigations are given in Table 5.

TABLE 5. ULTRAWEAK LUMINESCENCE OF SOME
IRRADIATED AND UNIRRADIATED OBJECTS [119].

Object	Dose, R	Background, pulses/min	Object, pulses/min	Tempera- ture, °C
mouse liver	0	20-30	100-150	38
"	700	20-30	160-250	38
lipids from dog liver	0	60-80	305-340	60
"	4200	60-80	1800-2050	60
mouse liver homogenate	0	20-30	60-70	50
olive oil	0	40-55	205-240	60
"	$3 \cdot 10^5$	40-55	400-440	60
oleic acid	0	60-80	860-1200	60
sunflower oil	0	20-30	60-90	60

The observed luminescence of tissues and lipids fell in the region 460-
500 mμ. Since the lipid extracts from the tissues themselves have an extremely
strong chemiluminescence during oxidation with oxygen [118, 119], it was natural
to assume that the spontaneous oxidation of lipids causes a luminescence of the
tissues, which therefore has a nonenzymatic character.
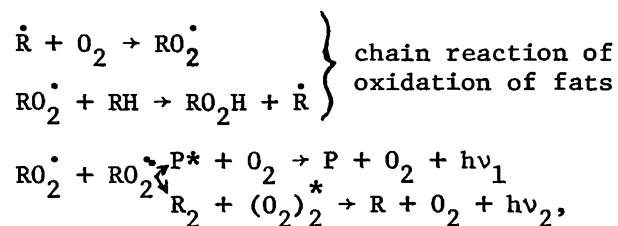
Luminescence Accompanying the Oxidation of Lipids

The problem of the luminescence mechanism accompanying the oxidation of
lipids in model systems was investigated in detail in a large series of studies
by B. N. Tarusov, A. I. Zhuravlev and their associates.

The following results were obtained in these studies.

1. In the course of autoxidation of unsaturated fatty acids (such as
oleic) or fats (such as vegetable oils) a luminescence was observed which was
sharply activated during heating. Since the autoxidation of fats transpires
via a chain mechanism similar to the hydrocarbon oxidation mechanism, it was
postulated that the mechanism of luminescence accompanying the oxidation of
fatty acids is similar to the mechanism of chemiluminescence accompanying the
oxidation of hydrocarbons, which was considered in the previous section.

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Luminescence therefore is associated with the formation of excited alde-
hydes or ketones forming during the recombination of peroxide radicals [69,
117]:

$$\dot{R} + O_2 \rightarrow R\dot{O}_2$$
$$R\dot{O}_2 + RH \rightarrow RO_2H + \dot{R}$$
$$\left. \right\} \text{ chain reaction of oxidation of fats}$$

$$R\dot{O}_2 + R\dot{O}_2 \left\{ \begin{array}{l} P^* + O_2 \rightarrow P + O_2 + h\nu_1 \\ R_2 + (O_2)_2^* \rightarrow R + O_2 + h\nu_2, \end{array} \right.$$

where $\dot{R}$ is the radical of the fatty acid; $R\dot{O}_2$ is the peroxide radical; RH is
the fatty acid; and P is a product of the reaction (ketone).

2. The introduction of ergosterol exerted no influence on the accumulation
of peroxides in oleic acid, but luminescence was intensified [69]. This can be
attributed to the sensitized fluorescence of ergosterol, which has a greater
yield of luminescence than ketones:

$$P^* + A \rightarrow P + A^* \rightarrow P + A + h\nu,$$
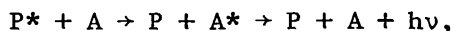
where A is a molecule of the activator. A similar picture, as mentioned in the
preceding section, is observed during the addition of dibromanthracene and oth-
er activators to a solution of an oxidating hydrocarbon [11, 20, 21].

3. The addition of anti-oxidants to oleic acid ended the further forma-
tion of peroxides. At the very moment of addition of some anti-oxidants, such /32
as β-mercaptopropyl amine, β-mercaptoethyl amine, adrenalin or lecithin, there
was a burst of luminescence [61, 66, 69, 117]. This can be attributed to reac-
tions of the type

$$ROO^{\cdot} + In^{\cdot} \rightarrow ROOIn^*,$$
$$ROO^{\cdot} + InO_2 \rightarrow P^*,$$
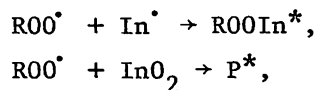
where $In^{\cdot}$ is the radical of an inhibitor of chain oxidation. However, it should
be noted that in our opinion the simple statement of the fact that a luminescence
burst occured does not make it possible to fully formulate the mechanism of the
reactions transpiring at the time of the addition of anti-oxidants to the oxidiz-
ing fatty acid.

The addition of other anti-oxidants, such as β-ionol or α-naphthol, sup-
pressed luminescence [69, 117], probably because these compounds bound peroxide
radicals. The role of anti-oxidants, according to Zhuravlev's data, can also
be played by the antibiotics tetracycline and penicillin [58].

4. In the absence of oxygen, luminescence in oleic acid ceased. In this

case there also was no accumulation of peroxides and the already-formed perox-
ides were transformed into other products. After the admission of oxygen there
was a sharp burst of luminescence whose intensity exceeded by several times the
intensity of luminescence of the unevacuated samples [62]. This phenomenon of
"anomalous chemiluminescence" was attributed to the fact that a considerable
quantity of the R radicals is accumulated in a vacuum; after the admission of
oxygen these radicals are transformed into peroxide radicals ROO·, whose recom-
bination is accompanied by luminescence [62].

5. The addition of an alcohol extract of animal organs to the oxidizing
oleic acid led to a sharp intensification of luminescence [65, 70, 117] and to
a shift of its maximum [68], possibly due to the combined effect of tissue anti-
oxidants and chemiluminescence activators. This capacity for extracts to acti-
vate luminescence was different for different tissues from animals and man [61,
68, 69, 75]. It was dependent on the physiological state of the animal [64] and
changed in irradiated [68, 99, 119] and cancerous [67] tissues.

The interpretation of the results obtained in the investigation of lipid /33
chemiluminescence is extraordinarily complicated by the fact that the mechanism
of the very process of oxidation of these compounds remains far from fully clari-
fied. In addition, in a study of lipid luminescence, investigators have almost
always dealt with inadequately purified chemical compounds; and methods involv-
ing kinetics, which have been extremely successful in other cases, remained es-
sentially unused for this system. A method of free radical identification as
specific as electron paramagnetic resonance also was not used. Therefore, many
stages in those extremely detailed schemes which were proposed for explaining
the luminescence mechanism [69, 70, 117] seem to us to be based more on analogies
than on direct proof. The possibility that luminescence is associated with fac-
tors other than the recombination of peroxide radicals also is not excluded
[124]. Nevertheless, data on lipid chemiluminescence and on the role of perox-
ide radicals, anti-oxidants and activators in the development of luminescence
are of undoubted interest in a study of tissue luminescence if for no other rea-
son than that the latter contain many lipids, activators and natural anti-oxi-
dants [70].

Reactions Associated with the Effect of H_2O_2 on Biological Substrates

The reactions of chemiluminescence in biological systems, associated with
the recombination of peroxide radicals, are not characteristic of fatty acids
alone. Even a simple decomposition of hydrogen peroxide in an alkaline medium
is accompanied by luminescence [94]. During the catalytic decomposition of hy-
drogen peroxide in the system H_2O_2 + $FeSO_4$ there is a chemiluminescence which
is sharply enhanced if the role of a catalyst is not played by mineral salts of
iron, but instead by its combination with EDTA [193]. The luminescence accom-
panying the interaction of hydrogen peroxide with organic compounds at room tem-
perature was observed by a number of authors. For example, Strehler made a de-
tailed study of the luminescence accompanying the effect of hydrogen peroxide
on riboflavin, which was enhanced appreciably in the presence of iron and copper
salts [200]: these data were confirmed by the author and F. F. Litvin [32].
Chemiluminescence accompanying the effect of H_2O_2 on glycine was recorded by

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Gurvich, Yeremeyev and Karabchiyevskiy [164]. V. N. Benevolenskiy, N. N. Kosh-<u>/34</u>
cheyenko and V. A. Veselovskiy discovered luminescence accompanying the inter-
action of hydrogen peroxide and cysteine [9]. Chemiluminescence accompanying
the effect of hydrogen peroxide on proteins was observed by Stauff and Wolf
[196] and also by I. I. Sapezhinskiy and his associates [109]. Since all these
compounds are present in the tissues of animals and plants, it is natural that
luminescence is also observed when they are subjected to the effect of hydrogen
peroxide [37, 38, 100, 101], which attenuates with decomposition of the peroxide.

The role of enzymes catalyzing peroxide decomposition in tissues, however,
is unclear in chemiluminescence reactions. We discovered that luminescence is
observed during the effect of a crystalline peroxidase of horseradish on hydro-
gen peroxide in the presence of tyrosine (oxidation substrate) and during the
decomposition of the peroxide by a catalase [37, 38]. Stauff and Wolf [196]
later observed chemiluminescence during the interaction of peroxidase and hydro-
gen peroxide. However, these authors did not discover luminescence under the
influence of a catalase; in addition, in the decomposition of H_2O_2 by a catalase
no luminescence was observed in the experiments of G. A. Popov and B. N. Tarusov
[100, 101]. On the other hand, K. P. Kachanov and A. P. Purmal' [74] confirmed
our data. One of the reasons for such a difference in the results may be an
exceptionally high rate of the catalase decomposition of peroxide. For this
reason it is possible to record luminescence only if the process is sufficient-
ly prolonged (with small concentrations of catalase and considerable H_2O_2 con-
centrations) and if the luminescence recording begins immediately after the mix-
ing. This requirement apparently was not adhered to by all authors. For ex-
ample, in [101] "measurement of luminescence began 30–40 sec after mixing the
reagents in the reaction vessel" [101, p. 317]. It is not surprising that in
this case luminescence could not be registered under the influence of a cata-
lase, and homogenate chemiluminescence was not dependent on the concentration
of hydrogen peroxide: the latter had decomposed completely by the time of the
measurement.

G. I. Likhtenshteyn and A. P. Purmal' advanced the hypothesis that the
luminescence observed in the experiments of these authors during the interaction
of catalase with peroxide was not associated with the catalytic process itself
but with the oxidation of reactive groups of an apoenzyme. In actuality, chemi-<u>/35</u>
luminescence was not suppressed in the presence of $1.3 \cdot 10^{-1}$ M azide or fluoride
and persisted in an acid medium, that is, under conditions when the catalase
activity was suppressed [90]. However, we feel that the experiments carried
out to the present time are of a more qualitative than quantitative character
and since the luminescence occurs both under the influence of H_2O_2 on protein
[109, 196] and during the catalytic decomposition of H_2O_2 [94, 193], the problem
of the contribution of these two processes to luminescence in the presence of
catalase remains open.

Since the quantity of peroxides normally used in the model experiments
(about 1%) is never accumulated in the body, we get the impression that the
mechanisms considered here have no relation to the luminescence of tissues or
homogenates. However, this is not the case. There is certain indirect data

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indicating that the luminescence is not associated with the decomposition of the peroxide in itself, but with the subsequent formation of peroxide radicals from molecules of organic compounds present in solution. This is indicated by the absence of proportionality between the rate of peroxide decomposition and the intensity of luminescence both during the course of the reaction [94] and as a result of adding different catalysts. The catalytic decomposition of peroxide probably leads to the formation of the radicals $O_2^{\cdot}$ and $HO^{\cdot 2}$, which, interacting with organic compounds (admixtures), form the peroxide radicals of the latter. The recombination of these radicals is accompanied by chemiluminescence whose mechanism is close to the luminescence mechanism for lipids and aromatic hydrocarbons considered above. The formation of peroxide compounds in the organism may occur not only as a result of the influence of hydrogen peroxide, but also in the course of the catalytic oxidation reactions or under the influence of ionizing radiation. In particular, there are indications of the formation of nucleotides under the influence of ionizing radiation [188], the formation of peroxides during autoxidation of the SH groups of proteins, etc. [128]. Many of these processes are possibly accompanied by chemiluminescence, which is similar to the luminescence observed under the influence of hydrogen peroxide on the corresponding substrates.

Ultraweak Luminescence of Plant Tissues

The selection of a research object frequently plays a decisive role in a biological experiment. Plant shoots were a very suitable object for the study of luminescence accompanying the vital processes of plants. These are characterized by active metabolism and can be conveniently distributed over a large plane in front of the photomultiplier window in a wet chamber containing a gas medium of definite composition, and a regulated mineral supply and temperature.

The first investigations of plant shoot luminescence were made in 1954 by Colli, Facchini and their associates [144, 145]. The radiation intensity of the sprouts in the experiments of these authors exceeded by several times the dark background of the photomultiplier. It was dependent on the age of the shoots and the species of plants, and was not dependent on the preliminary illumination of the shoots. Sprout homogenates also had luminescence whose intensity was maximal at a pH of about 7.8 (about 100,000 quanta/sec·g, wet weight). Luminescence fell in the region 460-600 mμ with a maximum at about 550 mμ (estimated by measurments with light filters).

Later the luminescence of bean sprouts was observed in our experiments [32], and was investigated thoroughly and in detail using the sprouts of different plants in the studies of B. N. Tarusov, V. A. Veselovskiy, R. A. Gasanov, G. G. Mamedov and others [1, 2, 26, 44-48]. For recording luminescence, these authors also used photomultipliers cooled with liquid nitrogen and operating in a photon-counter mode.

The most important conclusion drawn as a result of these investigations was that the luminescence was closely associated with definite aspects of the vital activity of plants. First, it was observed only in the presence of oxygen;

with an increase of its partial pressure the luminescence was intensified until the oxygen content became equal to its content in the air (20%), and subsequently remained virtually unchanged [1, 26, 45, 46, 48]. Second, it specifically depended on temperature: the temperature optimum of luminescence was sharply expressed, lying at 37-42°C, and was dependent on the heat resistance of plants [2, 26, 45, 47]. Third, luminescence was suppressed by such inhibitors of plant /37 respiration as carbon dioxide and cyanide, although there still is doubt concerning the mechanism of these processes. With the drying out of shoots, and especially in the case of their death, for example, as a result of overheating, specific luminescence disappeared [44].

A change in pH exerted an appreciable influence: maximum luminescence usually was observed at pH 8-10, but in more acid or more alkaline media its intensity decreased sharply [44, 45]. The intensity of light emission by shoots was dependent on their age and was maximal in the growing parts.

All these facts indicate a relationship of luminescence with some specific biochemical mechanisms in plant cells; however, this is not simple to formulate. It has been postulated that the basis of luminescence is the enzymatic oxidation of tissue lipids, since the oxidation of oleic acid by a secreted preparation of lipooxidase led to rather strong luminescence [44, 46]; some data support a relationship between the luminescence of plants and the activity of ascorbinoxidase and autoxidating flavoprotein (V. A. Veselovskiy, reference in [44]). It is interesting to note that the radiation of shoots was sharply activated with the addition of KCN. This brings the luminescence of shoots closer to the biochemiluminescence of homogenates and a suspension of animal tissue mitochondria whose mechanism will be discussed in the next section.

Three basic problems face the investigator in studying the mechanism of ultraweak luminescence.

1. What biochemical reactions are a direct source of energy in chemiluminescence reactions?

2. What intramolecular processes lead to the energy transformation from the chemical reaction to the energy of the excited state of a molecule?

3. What molecules are responsible for the emission of a quantum?

These problems will be discussed in this and subsequent sections.

Relationship Between Ultraweak Luminescence from Homogenates and Mitochondria with the Accumulation of an "Oxidized Product"

In the first investigations made using animal tissue homogenates it was not possible to detect luminescence under physiological conditions, that is, at 38°C. Liver homogenates from nonirradiated rats had a quite strong luminescence only at 50-60°C [118]. However, this was the situation only in the case of freshly prepared homogenates. With progressing incubation, in the liver, muscle and brain homogenates of a rat, prepared in a Krebs-Ringer solution, there was gradual development of luminescence considerably exceeding the dark background [38]. Its intensity attained a maximum 20-40 minutes after incubation in the presence of oxygen and then again decreased. The luminescence from the rat liver homogen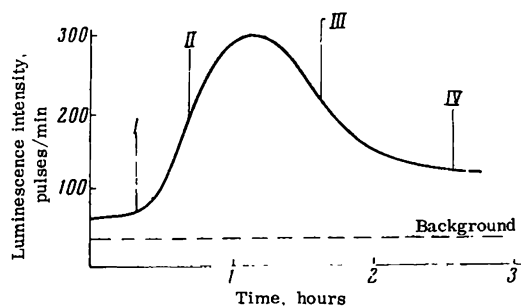ates under physiological conditions was discovered simultaneously and independently by Stauff and Schmidkunz [193] who also demonstrated that the replacement of oxygen by nitrogen leads to an attenuation of luminescence. /39 A more detailed study of individual stages of luminescence in tissue homogenates and tissue pulp [40] reveals that it is possible to define four developmental phases which were caused by the successive alternation of four processes. In each phase the intensity of luminescence was limited by a definite process (Figure 3).

Figure 3. Four Phases of Development of Ultraweak Luminescence in Homogenates [40]. I = Elution; II = Accumulation of "Oxidized Product" (XO_2); III = Expenditure of XO_2 Accompanying Luminescence; IV = Residual Luminescence.

Phase I. This is a latent period, probably caused by elution of water-soluble substances from the cells or from their fragments. In the pulp, phase I was expressed more strongly than in the homogenate [40], but as we will see later, it is virtually absent in a suspension.

Phase II. There is a gradual inten-

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sification of luminescence which apparently reflects the accumulation of some "oxidized product"

$$X + O_2 \rightarrow XO_2.$$
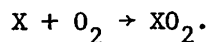
In the absence of oxygen, when the formation of an "oxidized product" is impossible, no luminescence was observed, but at the same time the first phase (elution) occurred: with initiation of the passage of oxygen through a homegenate which first had been incubated under anaerobic conditions, the luminescence developed vigorously without any latent period (Figure 4). It is possible that the first phase includes not only elution, but the transpiring of chemical reactions making conditions ready for the oxidation of the product X by molecular oxygen.

Phase III. A decline in luminescence. /40 In the simplest case the cause of the luminescence may be the consumption of the "oxidized product" as a result of some chemical reactions, some of which may be accompanied by luminescence:

$$XO_2 + Y \rightarrow Z + h\nu.$$
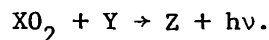
Phase IV. Residual luminescence, attenuating very slowly with time and caused by some less specific oxidation process of the substrates, present in great excess. This phase usually sets in 2-3 hours after the incubation of the homogenate at a temperature of 37-39°. Since heating only accelerated all phases of luminescence development, there was no change essentially in the total quantity of light emitted by the sample during the time of increase and decrease in luminescence and the conclusion was drawn that the supply of matter X, whose transformation can cause luminescence, is limited. Therefore, the more intense the luminescence, the less persistent it is. In addition, heating did not lead to an increase in the emission efficiency of a quantum during a chemical process (phase III), but simply accelerated the reactions accompanying luminescence [40].
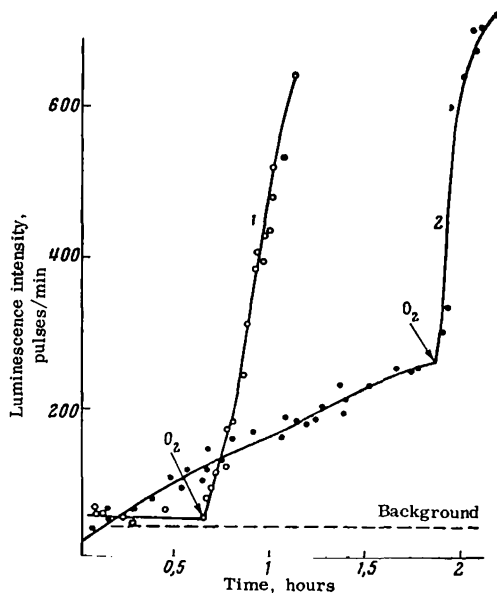

Figure 4. Influence of Oxygen on the Development of Luminescence in Liver Homogenates [40]. The Arrows Indicate the Oxygen Admission Times. Curves 1 and 2 = Results of Two Experiments.

It is entirely obvious that with such a situation it is senseless to speak of a temperature coefficient for luminescence as a whole. At the same time it is possible to determine the influence of temperature on the rate of individual phases by selecting definite segments on the curve of luminescence development

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corresponding to those times at which a definite limiting reaction is the "bottleneck" of the entire chain of processes. In particular, we investigated the temperature dependence of the second phase of luminescence development -- the "oxidated product" formation phase, which in all probability was the basic stage of energy accumulation for the future chemiluminescence quantum. The activation energy of this process was determined by measuring the curves of luminescence development for liver homogenates from one rat at 37, 39, and 41°C. In the region of the steepest ascent of these curves we drew tangents. The tangent of the angle of slope of the tangent to the x-axis was used as the rate of accumulation of the product XO_2 (phase II) at a given temperature [40]. In the selected segment of the curve the other reactions apparently exerted a minimum influence on the kinetics of luminescence development. The activation energy of the phase II reaction, computed using the Arrhenius equation, was 57.6 Cal/mole for temperatures of 37-39°C and 55.2 Cal/mole for temperatures of 39-41°C [40].

We see that the determined activation energy was very high, considerably greater than in ordinary biochemical reactions (20-30 Cal/mole). We feel that this fact is very important; in particular, it explains the low intensity of the luminescence, on whose path nature has set up a high energy barrier. In overcoming this barrier, the molecule acquires, through the improbable process of oxidation, a large energy reserve which finally is emitted in the form of a chemiluminescence quantum. We will return to this problem in Section V.

Interesting results were obtained in a study of the effect of antioxidants and potassium cyanide on the luminescence of homogenates. Methylmercaptan and ascor- /42 bic acid, added to the homogenate after 30-40 minutes, suppressed the luminescence, apparently interacting with the "oxidized product" XO_2 [40]. With respect to potassium cyanide, its effect depended on the phase of luminescence development at the time when it was added. When 0.001 M KCN was added to the homogenates 10 minutes after the onset of incubation, there was a small burst of luminescence which then developed no further. However, if the KCN was added 30-180 minutes after incubation there was a particularly strong burst of luminescence, which was replaced by its sharply expressed suppression (Figure 5). The potassium cyanide apparently reacted in some way with the "oxidized product" XO_2 and this caused a burst; on the other
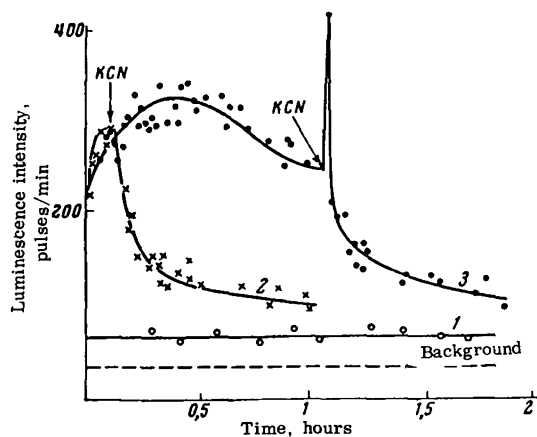

Figure 5. Influence of Potassium Cyanide on the Development of Luminescence in a Mitochondrial Suspension (Measurements Made by the Author Jointly with O. F. L'vova and Z. P. Cheremisina). KCN Added: 1 = Prior to Incubation; 2 = After 10 Minutes Incubation; 3 = After 70 Minutes Incubation. The Mitochondria were Isolated in 0.25 M Saccharose [116]. Composition of the Incubation Medium: 0.002 M K_2HPO_4; 0.005 M $MgSO_4$; 0.08 M KCl, 0.02 M ATP; 0.02 M Glutamic Acid; and 0.02 M Succininc Acid. KCN Concentration = 10^{-3} M.

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hand, it hindered the accumulation of XO_2.

Subsequent experiments revealed that of the four fractions of a cell obtained by centrifuging, the strongest luminescence per unit weight was observed in the mitochondria and microsomes and the weakest in the hyaloplasm and nucleus. Therefore, investigations of the mechanism of ultraweak luminescence were carried out later using primarily suspensions of mitochondria.

Accumulation of Lipid Peroxides and Ultraweak Luminescence

As already mentioned, B. N. Tarusov and his associates postulated that the luminescence of biological objects is associated with the nonenzymatic oxidation of lipids [119]. Certain data indicate that the luminescence of homogenates and mitochondrial suspensions which we observed is also associated with the formation of lipid peroxides.

First, lipids constitute a considerable part of the living tissue, including mitochondria. Twenty-five percent dry weight of mitochondria is accounted for by fatty acids, for the most part unsaturated: linoleic, linolenic and arachidonic [204]. Entering into the composition of the membrane structures of mitochondria and cells, these fatty acids cannot help being oxidized and such oxidation is accompanied, as already mentioned, by the formation of peroxide radicals and luminescence.

The formation of fatty acid peroxides occurs during the incubation of mitochondria in the presence of iron salts, ascorbic acid, cysteine or reduced glutathione [159, 169], as usually is judged from the increase in the concentration of malonic dialdehyde (MDA). Iron ions catalyze the oxidation of fatty acids [214]. Ascorbic acid possibly serves as a reducer, catalytically regenerating active bivalent iron from trivalent iron, which forms in the course of the reaction [184]. It also may be an initiator of a chain process, forming free radicals in the course of autoxidation [174] or during interaction with proteins [106]. In addition, ascorbic acid and cysteine facilitate the release of iron from the structure of mitochondria [159].

Ye. S. Neyfakh and his associates (personal report) have demonstrated that in a mitochondrial suspension there is a parallel formation of peroxides in the presence of ascorbic acid and the development of luminescence. We confirmed /44 these data. Figure 6 shows the results of experiments in comparing luminescence development and the accumulation of malonic dialdehyde. This figure clearly shows that the addition of ascorbic acid strengthened the intensity of the luminescence and increased the accumulation of malonic dialdehyde. After the accumulation of MDA ceased, the luminescence began to attenuate. The addition of bivalent iron salts to mitochondria also was accompanied by an enhancement of luminescence [41]. EDTA and KCN, bonding iron and restraining the accumulation of peroxides in fatty acid suspensions [214] and mitochondria [168], simultaneously completely suppressed the development of luminescence [39, 41].

Data on the influence of EDTA on the luminescence intensity of a mitochon- /45 drial suspension one hour after incubation are given below.
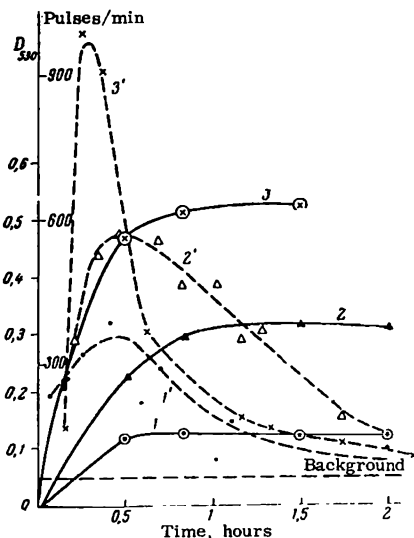
Figure 6. Accumulation of Malonic Dialdehyde and Development of Luminescence in a Mitochondrial Suspension in the Presence of Different Concentrations of Ascorbic Acid. $1,1' = 10^{-5}$ M; $2,2' = 10^{-4}$ M; $3,3' = 10^{-3}$ M [41]; 1, 2, 3 = Optical Density at 530 mµ; $1'$, $2'$, $3'$ = Luminescence, Pulses/ Min. Composition of Incubation Medium Same as in Caption to Figure 5.

EDTA concentration, moles	Pulses, min
0	268
10^{-6}	340
10^{-5}	76
10^{-4}	64
Dark background	48

All these facts indicate that the product XO_2 postulated above is formed as a result of fatty acid oxidation. However, it is not the final result of oxidation (malonic dialdehyde) but an intermediate compound, such as the peroxide radical, which later is transformed into MDA. The luminescence therefore ceases when the MDA concentration ceases to increase (see Figure 6).

The problem of the nature of the product Y (in the formula $XO_2 + Y \to Z + h\nu$) is not so definite. According to the initial ideas developed by A. I. Zhuravlev and his associates, the luminescence in lipids (and probably in the tissues) caused a recombination of peroxide radicals with tissue anti-oxidants [65]. However, it still is not entirely clear exactly what compounds play the role of anti-oxidants. In model systems the role of Y apparently can be played by KCN (see Figure 5).

Relationship Between Ultraweak Luminescence in Mitochondria and Oxidative Phosphorylation

Since the luminescence from homogenates in a salt medium develops simultaneously with swelling processes, mitochondrial lysis and nonenzymatic oxidation of biosubstrates, we initially called it "destructive", emphasizing by this term that it is death and not normal functioning of the cells which is accompanied by light emission [38]. This also was confirmed by the fact that intact mitochondria, separated by the standard method in 0.25 M saccharose in the presence of EDTA, did not luminesce during incubation in a salt medium containing an adenylic system and oxidation substrates*. /46 However, a more detailed investigation of mitochondrial luminescence in the absence of EDTA revealed that the mechanism of the processes leading to luminescence was by no means as simple as it appears at first glance.

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*Apparently other investigators also failed to detect luminescence in intact mitochondria. For example, Steele writes: "In attempting to observe mitochondrial chemiluminescence in rat liver we observed an emission of light with the addition of hydrogen peroxide to the preparation" [197, p. 529].

The experiments revealed first that the mechanical damage of the mitochondria during their separation led to an attenuation of the biochemiluminescence, which, for example, was considerably stronger if the homogenate was obtained using a glass rather than a metal homogenizer. In the latter case the luminescence of the homogenates was appreciably weaker than the pulp from this same quantity of tissue [40]. If the mitochondria were separated in a natural state and then frozen and thawed, the intensity of luminescence in them decreased sharply. Heat-denatured mitochondria had no chemiluminescence [40].

Thus, in order to observe quite strong luminescence it was necessary to begin the experiment with undamaged, normally functioning mitochondria. However, since the basic function of mitochondria in the cell is the formation of ATP in oxidative phosphorylation, we naturally were interested in the problem of what influence may be exerted on mitochondrial luminescence by oxidative phosphorylation. For this reason a study was made of the influence exerted on luminescence by such factors as oxygen, the respiration substrate, the adenylic system, osmotic pressure, calcium and magnesium ions, and different poisons. We will briefly discuss the results [39, 41].

First it was found that two factors necessary for normal oxidative phosphorylation in isolated mitochondria -- oxygen and the adenylic system -- also are absolutely necessary for the development of luminescence. Without the admission of oxygen or without addition of ATP (or ADP) it was impossible to observe any luminescence [39]; it also attenuated appreciably in the absence of respiration substrates: glutamic or succinic acids [39, 41]. Oxidative phos- /47 phorylation is very sensitive to a change in pH and osmotic pressure [116]. This also applied to the luminescence of mitochondria. In a hypotonic solution no luminescence developed in the mitochondria at all, despite the fact that all the remaining necessary conditions were present. As demonstrated by the experiments of O. F. L'vova and Z. P. Cheremisina in our laboratory, luminescence was maximal at pH 7.5. At pH 5.9 and 9.5 luminescence was appreciably attenuated.

It is known that the replacement of magnesium ions by calcium ions in a medium added to a mitochondrial suspension intensifies free oxidation in mitochondria to the detriment of oxidation associated with phosphorylation [116]. With the presence of calcium ions in a incubation mixture, the development of luminescence was considerably poorer than in the presence of magnesium ions. A study of the effect of poisons on luminescence development in intact mitochondria also indicates a relationship between luminescence and oxidative phosphorylation. Not only cyanide, but a dissociating poison -- 2,4-dinitrophenol -- completely suppressed luminescence development at a concentration of $2 \cdot 10^{-5}$ M, that is, in such a concentration at which its nonspecific effect as, for example, a quencher of fluorescence, still has not begun to exert an influence [41]. Sodium amobarbital, at a concentration of $2 \cdot 10^{-3}$ M, blocking the path to the flux of electrons in the NAD-flavioprotein sector [176], suppressed luminescence development in mitochondria, at least in the first hour of incubation [41]. Finally, the addition of hexokinase and glucose to the system, activating phosphorylating oxidation, led to stronger accumulation of fatty acid peroxides (to be more precise, MDA) and luminescence (Figure 7).

All these data indicate a relationship between mitochondrial luminescence and oxidative phosphorylation, and initially we even thought that the formation of excited molecules in mitochondria occurs as a result of direct oxidation by molecular oxygen of parts of the electron transport chain [36]. However, many facts contradict this, especially the very fact of luminescence development with time. On the other hand, since luminescence is associated with an accumulation of fatty acid peroxides, the problem can be formulated more precisely: how is the formation of lipid peroxides, accompanied by luminescence, associated with oxidative phosphorylation?

Apparently, it now is possible to advance two alternative explanations of /48 this phenomenon. The basis for the first of these is the fact that the swelling of mitochondria (for example, under the influence of thyroxine) occurs only under conditions of an electron transfer along the respiratory chain [175]. On the other hand, in the case of swelling under the influence of ascorbic acid, there is a release of iron ions from the mitochondria [159], and as a result, the formation of lipid peroxides [196], which is accompanied by luminescence.
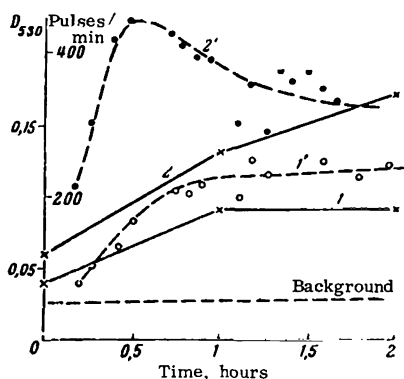

Figure 7. Influence of Hexokinase on the Accumulation of Malonic Dialdehyde and the Luminescence of Mitochondrial Suspensions [41]. 1,1' = Without Hexokinase; 2,2' = in Presence of Hexokinase, 0.1 mg/ml; 1, 2 = Optical Density at 530 mμ; 1', 2' = Luminescence, Pulses/min. Composition of Incubation Medium Same as in Caption to Figure 5.
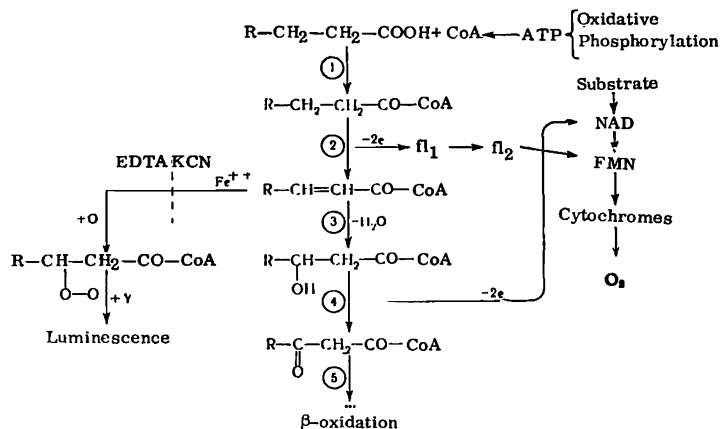
Thus, according to this hypothesis, the sequence of processes in swelling mitochondria can be represented in the following way:

Electron transport $\rightarrow$ swelling $\rightarrow$ release of Fe^{++} and fatty acid labilization $\rightarrow$ accumulation of peroxides $\rightarrow$ luminescence.

However, this hypothesis is not without its shortcomings. First, it does not solve the problem of the mechanism for the influence of the electron transport system on swelling. Second, the swelling of mitochondria in the presence of ascorbic acid possibly is not the cause, but the result of lipid peroxide forma- /49 tion. Therefore, another hypothesis has been advanced -- that the electron transport system is associated directly with fatty acid activation and peroxide formation, whose accumulation in turn cause the swelling of the mitochondria and the initiation of oxidative phosphorylation [41].

The central point in this hypothesis is that the formation of peroxides occurs not simply from fatty acids, but from activated (by coenzyme A) unsaturated fatty acids which form as one of the links of the lipid β-oxidation chain (see diagram).

This hypothetical model shows that activation of the fatty acid by coenzyme A (reaction 1) is necessary for the onset of β-oxidation and for the formation of oxides. Oxidative phosphorylation is the energy source for this activation.

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R—CH2—CH2—COOH+ CoA ◄— ATP { Oxidative Phosphorylation

Substrate
↓
NAD
↓
FMN
↓
Cytochromes
↓
O_2

(1)

R—CH2—CH2—CO—CoA

(2) $\xrightarrow{-2e}$ fl$_1$ → fl$_2$

EDTA KCN

Fe^{++}

R—CH=CH—CO—CoA

(3) -H2O

+O

R—CH—CH2—CO—CoA
|
O—O | +Y

R—CH—CH2—CO—CoA
|
OH (4)

-2e

Luminescence

R—C—CH2—CO—CoA
‖
O (5)

β-oxidation

The suppression of oxidative phosphorylation therefore leads to a suppression of luminescence, whereas the addition of oxidation substrates facilitates its development (see above). The unsaturated fatty acids, activated by coenzyme A, which forms as one of the intermediate products during β-oxidation, may either enter into enzymatic oxidation reactions (reactions 3–5 in the model) or are /50 oxidized directly by molecular oxygen; the latter process is catalyzed by iron ions and is accompanied by luminescence.
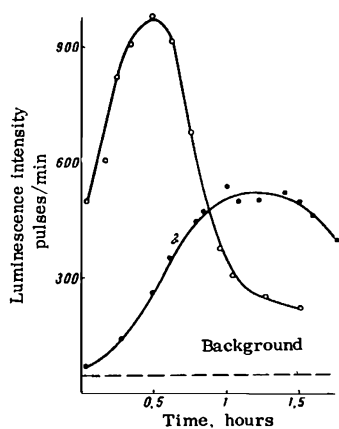

Figure 8. Luminescence of Natural and Denatured Mitochondria in the Presence of 10^{-4} M Ascorbic Acid [41]. 1 = Natural Mitochondria; 2 = Mitochondria Denatured by Heat. Composition of Incubation Medium the Same as in Caption to Figure 5, but without ATP.

It is possible that these two transformation pathways for activated unsaturated fatty acids (enzymatic β-oxidation and nonenzymatic peroxide formation) are competitive. In this case the factors facilitating the accumulation of NAD-N should slow down the enzymatic oxidation of unsaturated fatty acids (reaction 4) and thereby intensify the nonenzymatic formation of peroxides and luminescence. It is interesting that in the presence of ascorbic acid a decrease of oxygen in the medium (which leads to an accumulation of reduced forms of the members of the electron transport chain) led to a sharp (by several times) intensification of peroxide formation [159]. The oxidation substrates, as already mentioned, activated the luminescence. In the liver microsomes, an addition of NAD-N led to activation of lipid peroxide formation; this process was enzymatic in nature [159].

The addition of ascorbic acid to mitochondria, from the point of view of this model, intensifies luminescence for two reasons: first, the ascorbic acid may serve as a respiratory substrate and intensify the enzymatic formation of activated unsaturated fatty acids; second, it catalyzes nonenzymatic peroxide accumulation. The data in

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Figure 8 are of interest in this connection: the addition of ascorbic acid to
thoroughly boiled mitochondria caused the development of luminescence, which,
however, was more intense and developed considerably more rapidly if natural
mitochondria were used.

It therefore may be assumed that the relationship between oxidative phos-
phorylation, lipid oxidation, luminescence and swelling of mitochondria can be
represented in the form of the model:

Oxidative phosphorylation → β-oxidation of lipids → accumulation of per-
oxides and luminescence → swelling of mitochondria → release of oxidative phos-
phorylation.

In this chain of processes we still do not understand the mechanism for
the influence of peroxide formation on swelling, although the phenomenon itself
is well known. Important observations associated with this problem were made
with the addition of ATP, EDTA and serum albumin to swelling mitochondria. In
the first stages of swelling (caused, for example, by thyroxine) ATP, as is
well known, causes a contraction of the mitochondria [175, 176], whose membrane
contains protein resembling actomyosin [95]. However, the addition of ATP to
mitochondria, swelling in 30 minutes in the presence of cysteine and Fe^{++}, did
not cause their contraction; the addition of EDTA (binding iron) put an end to
the accumulation of fatty acid peroxides, but no longer could stop the continu-
ing swelling of mitochondria. Contraction of the mitochondria occurred only in
a case [159] when there was simultaneous addition of ATP, Mg^{++} and serum albu-
min, binding the fatty acids [139] (and probably their peroxides). This makes
it possible to think that the peroxides suppress the activity of mitochondrial
contractile protein. Taking into account that in the activity of myosin the
decisive role is played by SH groups and that the latter actively react with
lipid peroxide radicals [6, 60, 63, 69] it may be surmised that the formation
of peroxides influences swelling if for no other reason then that the reverse
process — contraction — is delayed. However, at the same time the lipid
peroxide formation may facilitate the emergence of fatty acids from the mem-
branes (which also accompanies the processes of mitochondrial swelling [175])
and change in the structure of these membranes.

The swelling and contraction of mitochondria is an important aspect of oxi-
dative phosphorylation regulation [175]. Oxidative phosphorylation "triggers"
reactions leading to swelling, and swelling leads to its release. In the course
of phosphorylation, ATP is formed and under its influence there is a contrac-
tion of the mitochondria. In short, the mitochondria operate as a complex self-
regulating system with a negative feedback, which caused Lehninger to call them
a "cybernetic machine" [175]. The change in the membrane permeability of the
mitochondria is also importnat for the regulation of processes transpiring in
protoplasm and even in the nucleus; for example, glycolysis [96]. All this
shows that lipid peroxide formation in mitochondria may be something more than
simply a secondary process of lipid β-oxidation, as can be demonstrated at
first glance. With further development of the studies in this direction, mea-
surement of ultraweak luminescence may be an extremely sensitive for the forma-
tion of peroxides (or peroxide radicals). Moreover, the luminescence possibly

accompanies the most important reaction into which peroxide enters: the interaction of the peroxide radical with the reactive group of the enzyme.

Since the processes of oxidative phosphorylation, swelling of mitochondria and formation of peroxides to some degree transpire in normally functioning tissues, it must be expected that the vital processes of the latter also should be accompanied by ultraweak luminescence. This phenomenon was discovered for the first time by B. N Tarusov, A. I. Polivoda and A. I. Zhuravlev [98, 119]. The authors measured the luminescence of the liver in living mice in a clever experiment, placing the animals with opened abdominal cavities opposite the window of a cooled photomultiplier. It is interesting to note that the undamaged tissue (liver) had considerably stronger luminescence than a homogenate under these same conditions [119]. Stauff and Reske observed the radiation from a yeast cell suspension when oxygen was passed through it. The intensity of this luminescence increased with the age of the culture. It was the cells themselves which had the luminescence, not the liquid phase of the culture [191]. We already have mentioned the experiments of N. A. Troitskiy, S. V. Konev and M. A. Katibnikov in which a Geiger counter was used for detecting the ultraviolet luminescence of yeast cells on agar [122]. Later these same authors ob-/53 served the luminescence of yeasts in the visible region of the spectrum [82]; recently S. V. Konev, T. I. Lyskova and G. D. Nisenbaum have demonstrated that cells in a synchronous culture of Torula utilis had an ultraviolet radiation (band, 250-400 mμ with a maximum at 330 mμ) whose maximum was observed at a time corresponding to the preparation of cells for division [81].

The luminescence from plants already has been mentioned. The clear relationship between the biochemiluminescence and the vital processes of an organism, the role of lipooxidase, ascorbin-oxidase and KCN in this process, and some other facts indicate that the mechanism of plant luminescence may have much in common with the luminescence of mitochondria considered in this section.

Somewhat to one side is yeast cell chemiluminescence induced by light. This luminescence was discovered by the author in collaboration with F. F. Litvin and T'an Man-chi [37-38]. It was closely related to the life processes of yeasts and possibly was caused by the photochemiluminescence of flavin compounds. However, its specific mechanism has remained uninvestigated.

In conclusion, a few words should be said concerning experiments in which luminescence was initiated by the preliminary irradiation of tissues, homogenates and lipids by ionizing radiation [64, 66, 118-122] or by the addition of hydrogen peroxide [101]. In all these cases the effective agent could either give rise to a lipid oxidation chain reaction or destroy the anti-oxidants which prevent the development of the chain. The data obtained until now, in our opinion, do not make it possible to give a sound preference to any one of these possibilities.

Thus, the most investigated and universal mechanism of luminescence from plant and animal tissues may be considered the chemiluminescence of lipid peroxides, whose accumulation is associated with oxidative phosphorylation in mitochondria. However, there possibly are other still unstudied biochemical reactions which supply the energy for cellular chemiluminescence.

V. LUMINESCENCE ACCOMPANYING REVERSE PHOTOCHEMICAL REACTIONS AND THE GENERATION MECHANISM OF A QUANTUM IN BIOLOGICAL SYSTEMS

As in the investigation of photochemical reactions, in the study of chemiluminescence we can distinguish chemical and physical approaches to the problem. On the one hand, an investigator may be concerned with the problem of what chemical or biochemical reactions in a particular system are accompanied by luminescence; it is just this sort of work which for the most part has been discussed thus far. On the other hand, it is fitting to discuss the problem of what mechanisms are involved in the final, physicochemical stage of chemiluminescence in which the chemical process leads to the appearance of an excited molecule.

Chemiluminescence is a process the reverse of the photochemical process. In particular, this applies to reactions directly related to the emission of a quantum. Therefore, one of the experimental approaches to studying the generation of a quantum of ultraweak luminescence may be the study of chemiluminescence accompanying reverse photochemical reactions. Now we will consider some results of such investigations, limiting ourselves for the most part to biochemical examples.

Recombination Luminescence in Frozen Solutions of Aromatic Amino Acids and Proteins

The simplest photochemical reaction now known is the photoionization of organic compounds:

$$X + h\nu \rightarrow X_.^+ + e^-.$$
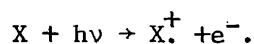
This results in the formation of the positive ion-radical $X_.^+$ (cation radical) and an electron (e^-), captured by the molecules of the medium (solvated elec- /55 tron). This reaction is reversible, the products are unstable, and it is possible to detect them at room temperature only by using the pulse photolysis method. This method was used by Grossweiner, Swenson and Zwicker for demonstrating that the lifetime of a hydrated electron at room temperature is 25-50 μsec, whereas the radicals of organic compounds, including aromatic amino acids, are retained for 100 μsec after irradiation [162, 163]. At the temperature of liquid nitrogen the solvated electrons are caught by traps in a frozen solvent and become stabler. Their recombination with radicals under these conditions is accompanied by luminescence. An example of such recombination luminescence is the prolonged afterglow of frozen solutions of aromatic amino acids and proteins after ultraviolet irradiation at 77°K [151]. This luminescence is activated during the heating of samples (low-temperature thermoluminescence) [42, 43, 104, 105]. The depth of the traps is different in solutions of amino acids and proteins [42, 105] and in crystals of tyrosine and tryptophan in these same cases [161, 211].

The results of further investigations of the mechanism of reaction $X_.^+ + e^-$ $\underset{\rightarrow}{\Delta}$ X + hν can be understood better if we consult the model shown in Figure 9.
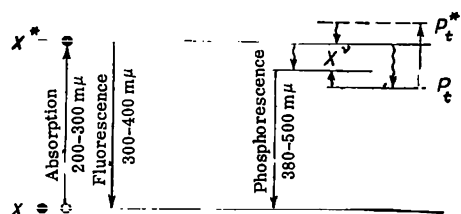
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Figure 9. Diagram of Electron Transitions Accompanying Photoionization of Aromatic Amino Acids and with Luminescence Induced by Heat and Light. X is the Ground State of the Molecule; X^* is the Singlet Excited State; X^ν is the Triplet State; P_t is the Ground State of the Product P (Level of Electron Trap); P_t^* is the Excited State of P_t; $X \to X^* \to P_t$ is Photoionization; $P_t \to P_t^* \to X^* (X^\nu) \to$ X is Light-Induced Luminescence; $P_t \to X^\nu \to$ X is Thermoluminescence. Arrow Pointing Upward -- Absorption of a Quantum; Arrow Pointing Downward -- Emission of a Quantum; Wavy Arrow -- Nonradiative Transition.

As demonstrated by investigations of the direct photochemical reaction of aromatic amino acid photoionization [3, 4, 29, 209, 210], this process occurs through the singlet excited state of the molecule:

$$X + h\nu \to X^* \to \underbrace{X_\cdot^+ + e^-}_{P_t},$$
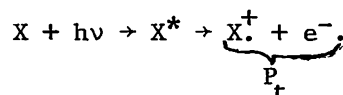
The reverse reaction leads to the formation of a molecule of the initial amino acid in the excited singlet X^* or triplet X^ν state in dependence on the factor responsible for the recombination. If the recombination was activated by heat (prolonged afterglow and thermoluminescence of amino acids), the luminescence spectrum, evaluated using light filters, is close to the phosphorescence spectrum; there was no radiation in the spectral region of fluorescence [104, /56 210]. The kinetics of luminescence attenuation at 77°K after brief heating of the irradiated sample to 90°K also coincided with the kinetics of attenuation of ordinary phosphorescence [4, 29, 210]. All this indicates that the recombination of a cation radical and a solvated electron, activated by heat, occurs through the triplet excited state of an aromatic amino acid:

$$X_\cdot^+ + e^- \xrightarrow{\text{heating}} X^\nu \to X + h\nu_{phos},$$
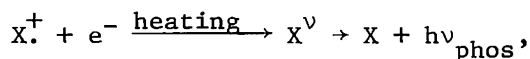
where $h\nu_{phos}$ is a phosphorescence quantum.

A different picture is observed in the case of illumination of a frozen solution containing solvated electrons and the cation of a radical. In this case there was "anti-Stokes fluorescence" induced by visible light which attenuated with expenditure of the photoproducts $X_\cdot^+$ and e^- [2, 3, 4, 11]. Therefore, with the activation of recombination by visible light there is a formation of amino acid molecules in a singlet excited state:

$$X_\cdot^+ + e^- \xrightarrow{\text{illumination}} X^* \to X + h\nu_{fl},$$
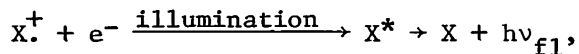
where $h\nu_{fl}$ is a fluorescence quantum. These processes also are represented /57

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schematically in Figure 9. It goes without saying that induced fluorescence was accompanied simultaneously by "induced" phosphorescence as a result of a singlet-triplet transition in an amino acid molecule:
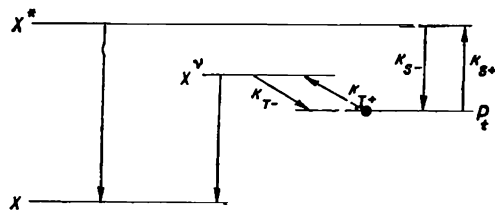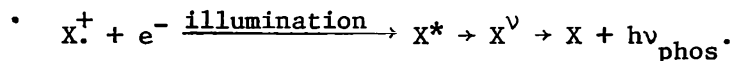
$$\overset{\bullet}{X^+} + e^- \xrightarrow{\text{illumination}} X^* \to X^{\nu} \to X + h\nu_{phos}\,.$$

Figure 10. Electron Transitions to Singlet (X*) and Triplet (X$^{\nu}$) Levels From Trap (P$_t$). Explanations in Text.

The problem arises as to what caused the transition of a solvated electron at different levels in a molecule. Let us consult Figure 10. If the transfer of an electron from the trap did not require expenditures of activation energy, the constants of the rate of electron transition to the singlet or triplet level (k$_S$ and k$_T$) would be determined by the corresponding oscillator strengths and would be limited only by the spectroscopic exclusion principles. The number of quanta emanating per unit time from the triplet state (I$_T$) in such a case is related to the number of quanta emanating from the singlet excited state (I$_S$) as

$$\frac{I_T}{I_S} = \frac{k_T}{k_S} \cdot \frac{\varphi_T}{\varphi_S}\,, \qquad \bullet \qquad (8)$$

where φ_T and φ_S are the probabilities of radiative transitions of an electron from the triplet and singlet excited states. We encounter such a situation during the recombination of $\overset{\bullet}{X^+}$ and e$^-$ under the influence of a light quantum which brings the electron to an energy level obviously higher than the singlet and triplet excited levels of the initial amino acid (see Figure 9). However, in the absence of illumination the expenditure of thermal energy is required for setting free an electron from the trap. Under these conditions equation <u>/58</u> (8) assumes the form:

$$\frac{I_T}{I_S} = \frac{k_T}{k_S} \cdot \frac{\varphi_T}{\varphi_S} \cdot e^{-\frac{\Delta E_T - \Delta E_S}{kT}}\,, \qquad (9)$$

where ΔE_S and ΔE_T are the activation energies of transition of an electron from the trap to the singlet or triplet level of the initial amino acid; k is the Boltzmann constant ($8.62 \cdot 10^{-5}$ eV/degree); T is absolute temperature.

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True, it must be noted here that equation (9) was derived for a simplified case: the probability of the transitions $X^* \to X$ and $X^\nu \to X$ was assumed to be considerably greater than the probability of photoionization $X^* \to P_t$ and $X^\nu \to P_t$. In actuality, however, the lack of measurements of the quantum yields of photoionization in aromatic amino acids for the time being does not make possible a precise estimate of the probability of these transitions. Nevertheless, it follows quite clearly from equation (9) that the contribution of the singlet and triplet states to recombination luminescence is determined by three factors:

the multiplicity factor k_T/k_S;

the quenching factor φ_T/φ_S;

the energy factor $e^{-\frac{\Delta E_T - \Delta E_S}{kT}}$.

These three values apparently play a decisive role in the chemiluminescence processes in general. Since in aromatic amino acids the distance between the singlet and triplet levels is 0.7-0.9 eV [30], then

$$\frac{I_T}{I_S} \cong \frac{k_T}{k_S} \cdot \frac{\varphi_T}{\varphi_S} \cdot 10^{-5}. \tag{10}$$

This means that if the fluorescent and phosphorescent components in luminescence induced by light have the same order or magnitude, that is,

$$I_T \approx I_S,$$

then in the case of thermoluminescence and prolonged afterglow the intensity of luminescence from the triplet level should exceed by 100,000 times the intensity of luminescence from the singlet level, which for practical purposes will not be /59 discovered, as is observed experimentally.

Thus, equation (9) shows that simple energy factors can cause the experimentally observed coincidence of the thermoluminescence and phosphorescence spectra. These same energy considerations could explain why many known chemiluminescence reactions are accompanied by the formation of triplet-excited molecules, provided the final stage of these reactions can be conceived in the form of the model in Figure 10. To what extent this is valid will be considered in the following sections.

The Relation Between Radiative and Nonradiative Transitions

Only an insignificant part of the solvated electrons recombines with emission of a thermoluminescence quantum and light-induced luminescence [3, 210].

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This is indicated, in particular, by the experimental results shown in Figure 11, which is a comparison of the curve of thermoluminescence of an irradiated solution of tryptophan, the change of intensity of light-induced luminescence and change of optical density at the absorption maximum of a hydrated electron during heating of the sample.
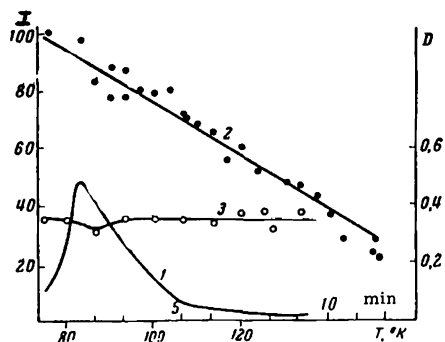

Figure 11. Release of Electrons from Traps during Heating of a Solution of Tryptophan in a 10 N Solution of NaOH Irradiated by Ultraviolet Light at 77°K [3].
1 = Thermoluminescence Curve; 2 = Intensity of Induced Luminescence; 3 = Absorption at 600 mμ (Absorption of Hydrated Electron); I = Intensity of Luminescence, Relative Units; and D = Optical Density.
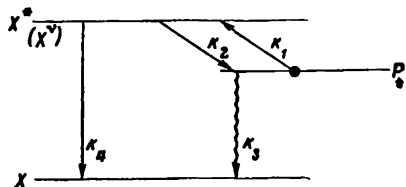
This figure clearly shows that the solvated electrons responsible for the thermoluminescence are the most thermolabile. The centers whose recombination under the influence of visible light is accompanied by radiation disappear (without radiation) at a higher temperature. Most of the solvated electrons are stable to 160°K, that is, to the time of melting of the matrix, and this recombination is not accompanied by luminescence either in itself (thermal activation) or under additional illumination (activation by light). Since a greater thermal stability means a greater depth of the electron traps, the observed phenomena indicate that the probability of a radiative transition during recombination is greater, the lesser the activation energy of the process. There is nothing surprising in this. We will turn to the diagram in Figure 12 for an explanation of the observed phenomena.

The probability of transition of an electron from the trap P_t to the ground state X, proportional to the rate constant k_3 of the transition $P_t \to X$, is dependent on the oscillator strength which corresponds to this transition and is not dependent directly on temperature. We will denote this transition probability by P_0:

$$P_0 \infty k_3. \tag{11}$$

Figure 12. Electron Transitions to Excited (X* or X$^\nu$) Levels and Ground State (X) from Trap (P_t). Explanation in Text.

The probability of transition of an electron to the excited (singlet or triplet) state (P_b) is not only proportional to the corresponding oscillator strength (constant k_2), but also is dependent on temperature in accordance with the equation

$$P_b \infty k_1 = k_2 \cdot e^{-\frac{\Delta E}{kT}}, \tag{12}$$

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where ΔE is the depth of the electron trap in relation to the excited state (difference of the P_t and X^* or P_t and X^ν levels). If we use φ_b to denote the probability that the transition of an electron from the excited level to the ground state is accompanied by the emission of a quantum (quenching factor), then

$$\frac{P_b}{P_0} = \frac{k_2}{k_3} \cdot \varphi_b \cdot e^{-\frac{\Delta E}{kT}} \qquad (13)$$

Since the quantum yield of chemiluminescence φ_{chemi} in this case is equal to the ratio of the number of emitted quanta to the total number of radiative and nonradiative transitions, that is,

$$\varphi_{chemi} = \frac{P_b}{P_0 + P_b}, \qquad (14)$$

and since usually $P_0 \gg P_b$, it is possible to write the final expression in the form:

$$\varphi_{chemi} = \varphi_b \cdot \frac{k_2}{k_3} \cdot e^{-\Delta E/kT}. \qquad (15)$$

It follows directly from this expression* that the quantum yield of recombination luminescence decreases sharply with an increase in the activation energy of the process (depth of the trap).

It is interesting to note two other corollaries from the diagram in Figure 12 and equation (15). The first of these is that the energy of the emitted quantum consists of the sum of the free energy of the reaction (difference between the P_t and X levels) and the chemiluminescence activation energy (difference between the P_t and X^* levels). This rule, which was formulated by Audubert [135], later was noted by a number of authors (see p. 12). However, being a simple corollary on the first law of thermodynamics, it does not take into account the limitations imposed by the second law, which does not make it possible for the thermal energy ΔE of molecular motion to be transferred entirely into radiation /62 energy. Equation (15) shows that the greater the contribution of thermal energy (activation energy) to the energy of an emitted quantum, the smaller the probability of its emission, that is, the lower the quantum yield of the chemiluminescence.

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*It should be noted that equation (15) was derived under the condition $k_2 \ll k_3$.

Since all the reasonings here are essentially founded on a thermodynamic basis to some degree they are applicable to chemiluminescence reactions independently of their mechanism. In a general case the energy levels on the diagram (see Figure 12) correspond to the thermodynamic potentials of the system in three different states: in the initial state (P), in a state when all the molecules

of the reaction product are electron-excited (X^* and X^ν), and in a case when all the molecules of the reaction product are in an unexcited ground state (X). The constants k_1 and k_3 characterize the rates of the competing chemical reactions.

These considerations explain how quanta can be emitted with an energy greater than the free energy released during the reaction. Moreover, they show that if the molecules which form as a result of the chemical reaction have corresponding energy levels, the luminescence for the same "gross" free energy of the process (less than the energy of a chemiluminescence quantum) may fall not only in the visible, but even in the ultraviolet region of the spectrum; however, the intensity of the chemiluminescence will have a tendency to decrease sharply with a decrease of wavelength.

Luminescence Accompanying Oxidation of Photoreduced Chlorophyll

The recombination of an electron and a cation radical probably is the simplest reaction accompanying luminescence. Another example of a model system where the mechanism of formation of a chemiluminescence quantum, although more complex, nevertheless can be explained, is the oxidation reaction of photoreduced chlorophyll by the oxygen of the air.

As demonstrated by A. A. Krasnovskiy and his associates, the chlorophyll in a solution of pyridine containing an electron donor, such as ascorbic acid, can be photoreduced:

$$X + h\nu \to X^* \xrightarrow{+ AH} X_\cdot^- + AH_\cdot^+ \to XH + A, \qquad\qquad \underline{/63}$$

where X is a chlorophyll molecule; AH is a molecule of ascorbic acid; XH is the product of final reduction of chlorophyll (secondary, "red" form); $X_\cdot^-$ is the primary "green" reduced form of chlorophyll (semiquinone), which is stable only at a low temperature (about $-40°C$) [83].

In a study made by F. F. Litvin, A. A. Krasnovskiy and the author it was demonstrated that in the reverse oxidation of photoreduced chlorophyll (after admission of oxygen) there is luminescence [84, 89]. This luminescence was intensified with the simultaneous addition of ammonia to the system, which, as is well known, catalyzes the transition of the secondary reduced form of the chlorophyll to the primary form.

It could be concluded that the luminescence was caused in fact by the oxidation of the primary photoreduced form of chlorophyll (ion radical). This was confirmed in an experiment in which a comparison was made between the kine-

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tics of luminescence attentuation, disappearance of the maximum at 525 mµ (absorption maximum of the secondary reduced form of chlorophyll) and the reduction of the chlorophyll maximum at 668 mµ after addition of ammonia and admission of oxygen into the solution of photoreduced chlorophyll. The 525-mµ maximum in these experiments disappeared quickly after the addition of ammonia and oxygen (transition of the secondary reduced form into the primary form), whereas the chlorophyll absorption at 668 mµ (transition of the primary reduced form into chlorophyll) was restored in a few minutes; attenuating luminescence was observed consequent with this process [89]. It also was possible to observe chemiluminescence in solutions irradiated at -40°C, that is, under conditions in which the secondary form of photoreduced chlorophyll is not formed at all. Thus, the scheme of the reactions leading to luminescence can be expressed by the equations:

$$XH \xrightarrow{\text{ammonia}} X\overset{-}{\cdot} + H^{+}$$

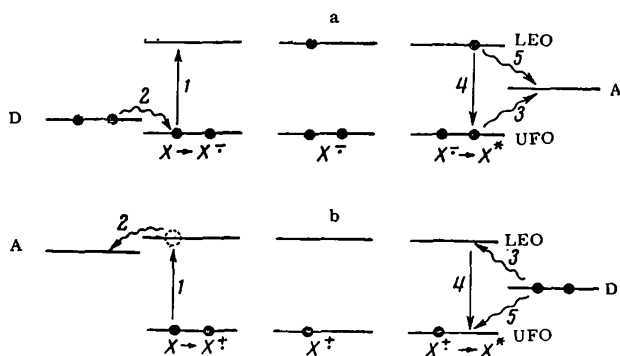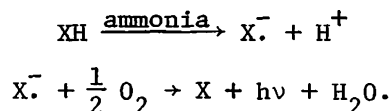$$X\overset{-}{\cdot} + \frac{1}{2} O_2 \rightarrow X + h\nu + H_2O.$$

Figure 13. Hypothetical Mechanism of Quantum Generation Accompanying Photochemiluminescence. a = "Donor" Luminescence Accompanying Reaction of Photoreduced Form (X⁻·) with Electron Acceptor (A); 1 = Absorption of a Light Quantum; 2 = Photoreduction of X; 3 = Electron Transfer to Acceptor A with Formation of Excited State X*; 4 = Emission of a Photochemiluminescence Quantum; 5 = Nonradiative Transition; b = "Acceptor" Luminescence Accompanying Reaction of a Photooxidized Form (X⁺·) with Donor Electron (D); 1, 4 and 5 = Same as in Figure 13,a; 2 = Photooxidation of X; 3 = Electron Transfer from Donor D with Formation of Excited State X*; LEO = Lower Empty Electron Orbital; UFO = Upper Filled Electron Orbital.

The scheme of formation of the excited state as a result of transition of an electron from photoreduced chlorophyll is shown in Figure 13,a. This scheme shows that the /64 mechanism for luminescence during the oxidation of a photoreduced form (Figure 13,a) seemingly is a mirror reflection of the mechanism of luminescence arising in the reduction of a photooxidized form (Figure 13,b); the latter process is similar to the low-temperature thermoluminescence of amino acids, considered in the preceding section. It is interesting that the luminescence of chlorophyll during the reactions accompanying photoxidation also has been discovered [89]. An excited state of the chlorophyll molecule is formed in both cases.

### Donor and Acceptor Luminescence Accompanying the Radical Recombination

The model in Figure 13 indicates that there are two different mechanisms for the emission of a chemiluminescence quantum. In the first case the formation of an   /65

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excited state of an "emitter" molecule occurs as a result of the transfer of an electron to an acceptor; in the second case -- as a result of its transfer from an acceptor. In accordance with the role performed by the "emitter" molecule, the first mechanism should be called the <u>donor</u> mechanism and the second should be called the <u>acceptor</u> mechanism. Luminescence accompanying the oxidation of photoreduced chlorophyll is donor luminescence. Luminescence accompanying the thermoluminescence of tyrosine and tryptophan is acceptor luminescence.

Studying the model in Figure 13,a, it can be seen that the transfer of an electron from the lower empty (to be more precise, "semi-empty") orbital of an "emitter" molecule (donor LEO) to the lower empty orbital of the acceptor (acceptor LEO) can occur in two ways: 1) donor LEO → acceptor LEO (Figure 13,a, <u>5</u>; 2) donor UFO → acceptor LEO (Figure 13,a, <u>3</u>) and donor LEO → donor UFO + hν (Figure 13,a, <u>4</u>).

In the first case the emission of a quantum does not occur in the direct transfer of an electron. In the second case a chemiluminescence quantum is emitted during two-stage transfer. The way in which the process occurs undoubtedly is dependent on the position of the acceptor LEO; the lower this level, that is, the higher the affinity of the acceptor to an electron, all other conditions being equal, the more probable is the radiative transfer. This reasoning is similar to that given in the preceding section. In this connection it is interesting to note that the oxidation of photoreduced chlorophyll by such electron acceptors as dyes, rather than by molecular oxygen, did not lead to chemiluminescence, although from the chemical point of view the chlorophyll molecule entered into the same reaction:

$$X^- + A \rightarrow X + A^-$$
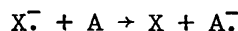
As already mentioned, oxygen is a necessary participant in almost all chemiluminescence reactions. According to the concepts developed here, this is related simply to its high affinity to an electron (low position of the electron acceptor LEO in Figure 13,a).

Comparison of the diagrams in Figures 12 and 13 shows that the *formation* mechanisms of an excited state during the recombination reactions of an electron and a cation radical (Figure 12) and during reverse reactions of photo-oxidation and photoreduction (Figure 13) have many features in common. It would be extremely attractive to go still further: e.g.,attempt to construct a /66 model of electron levels and transitions for other chemiluminescence reactions. Some hypothetical models of this type are shown in Figure 14. We feel that they are useful for a qualitative study of the phenomena.

First, Figure 14,a shows that the transfer of an electron in a system of molecules usually is not accompanied by the formation of excited states. This requires the interaction of a strong oxidizer and a strong reducer. The simplest case of a reaction of this type in solutions should be a reaction of the type

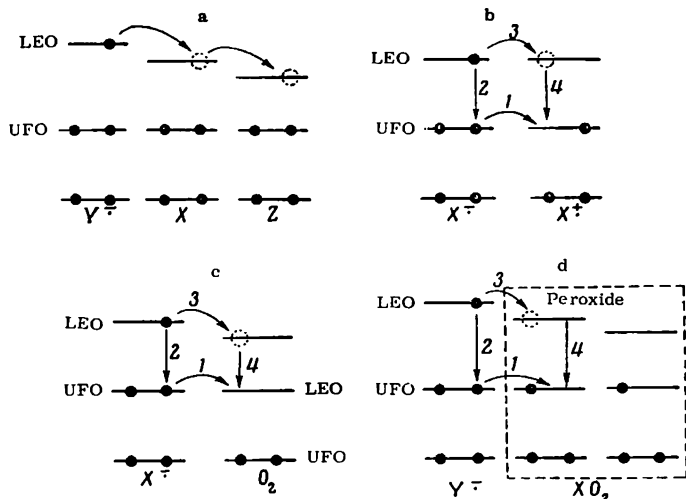$$X^+ + X^- \rightarrow X^* + X \rightarrow 2X + h\nu,$$

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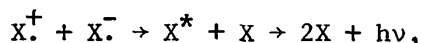
Figure 14. Hypothetical Mechanism of Quantum Generation Accompanying Chemiluminescence.
a = Electron Transfer Through System of Molecules Y, X, Z, not Accompanied by Luminescence; b = Formation of Excited State X^* Accompanying Recombination of Radicals $X^{\bar{\cdot}}$ and $X^{\dot{+}}$; 1,2 = Electron Transfers with "Donor" Luminescence; 3,4 = Electron Transfers with "Acceptor" Luminescence; c = Formation of Excited State Accompanying Interaction of $X^{\bar{\cdot}}$ Radical with Oxygen: 1-4 = Same as in Figure 14,b; d = Formation of Excited State X^* (During Transition 3) of Y^* (During Transition 1) Accompanying Interaction of $Y^{\bar{\cdot}}$ Radical with Peroxide XO_2; 1-4 = Same as in Figure 14,b; UFO = Upper Filled Orbital; LEO = Lower Empty Electron Orbital of Compounds X and Y in Ordinary (Oxidized) State.

whose model is shown in Figure 14,b. This process is improbable in ordinary chemical reactions because positive and negative ion radicals rarely are encountered simultaneously in the same system. However, it apparently occurs during electrolysis reactions of aromatic compounds [167] and luminol [172, 173]. The experiments carried out by Hercules [167] were extremely effective: during the passage of an electric current through a solution of anthracene or other polycyclic hydrocarbons in anhydrous, deaerated solvents there was luminescence at the cathode, which intensified during mixing of the liquid. Particularly bright luminescence formed at the time of passage of an alternating current, when the radicals $X^{\dot{+}}$, forming at the anode, could enter into contact with the radicals $X^{\bar{\cdot}}$, forming at the cathode. The effect of oxygen on this process is characteristic. The model in Figure 14,b shows that the energy necessary for the formation of an excited state during the reaction between $X^{\dot{+}}$ and $X^{\bar{\cdot}}$ is equal to zero; in accordance with equation (15), the quantum yield of the process when $\Delta E = 0$ can be of the order of unity. During the recombination of $X^{\bar{\cdot}}$ with oxygen the formation of X^* requires expenditures of activation energy and no longer can compete so successfully with the nonradiative transition ($X^* \rightarrow X$); in accordance with equation (15), the quantum yield of luminescence in this process should be appreciably less than unity (since $\Delta E > 0$). In actuality, in the experiments of Hercules the oxygen quenched the luminescence during electrolysis.

Unfortunately, the study of recombination luminescence ($X^{\dot{+}} + X^{\bar{\cdot}}$) in a purely chemical system is impossible for the time being due to the extreme instability of the cation radical $X^{\dot{+}}$. However, Chandross and Sonntag were able to come considerably closer to this idealized system by studying the luminescence arising

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during the recombination of an anion radical (X⁻) and the chlorine derivative 9,10-diphenylanthracene [141, 142]. The anion radical X⁻ was obtained by processing the diphenylanthracene with metallic potassium in a boiling anhydrous solvent. In the DPA⁻ and DPA-Cl₂ reaction there was a strong luminescence in its spectrum similar to the fluorescence of DPA, which should be expected on the basis of the model in Figure 14, b.

The most interesting aspect of the experiments of these authors is that luminescence was formed during the recombination of DPA⁻ and other electron <u>/68</u> acceptors: benzoyl peroxide, oxalyl chloride, and aluminum and mercury chloride. The authors conclude that "the luminescent reaction involves only the transfer of an electron from the DPA⁻ to the acceptor" ([141], p. 3180). In other words, the process is donor luminescence and has a mechanism corresponding to the model in Figure 13,a (transitions 3-4).

Thus, the concept of chemiluminescence as a molecular excitation process associated with the transfer of electrons to corresponding empty orbitals is supported by the study of luminescence accompanying reverse photochemical reactions, electrolysis, and chemiluminescence during reactions of anion radicals with oxidizers [34, 36, 141, 142, 167].

In conclusion, we will discuss briefly the mechanism of luminescence during a reaction in which one of the participants is a peroxide molecule (Figure 14,d). If a peroxide molecule XO₂ is found with a reduced form of any metabolite (such as with the ion radical Y⁻), an electron can move from the UFO of the metabolite Y⁻ to the UFO of the substance X. The subsequent transition LEO → UFO in a molecule of the substance Y will be accompanied by the emission of a chemiluminescence quantum. Thus, here the peroxide plays the role of a good electron acceptor (see the experiments of Chandross and Sonntag, mentioned above).

The diagram in Figure 14,d also shows that the luminescence accompanying reactions with the participation of peroxide, as in the preceding cases, can be of either the donor or acceptor type. Accordingly, the luminescence spectrum can be different: depending on the relative distribution of levels, it will coincide with the luminescence spectrum of the donor electron Y or the acceptor electron X, although from the chemical point of view the process in both cases will be the same.

There are three mechanisms of generation of a quantum: recombination of radicals (Figure 14,b), interaction of a radical and oxygen (Figure 14,c) and the interaction of peroxide with a strongly reduced compound (Figure 14,d). The latter probably occurs quite widely in biological systems. Again we will give examples of ultraweak luminescence accompanying biochemical reactions which may have this mechanism.

1) Chemiluminescence accompanying the effect of hydrogen peroxide on <u>/69</u> biologically important compounds: riboflavin [200], chlorophyll [178], proteins [109, 196], cysteine and mercaptan [9].

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2) Luminescence accompanying the effect of lipid peroxide radicals on antioxidants [61, 66, 69, 117].

3) Luminescence in mitochondria and homogenates [40]:

$$XO_2 + Y \rightarrow Z + h\nu.$$
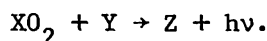
The role of Y can be played, in particular, by KCN [40]; XO_2 is a peroxide or a peroxide radical of lipids [40, 41].

4) Luminescence following irradiation of riboflavin in the presence of hydrogen peroxide [197].

5) The thermoluminescence of chloroplasts [84, 89, 123]: the illumination of chloroplasts is accompanied by the formation of lipid peroxides and during heating there is an interaction of the lipid with chlorophyll, accompanied by luminescence [123].

It is easy to see that a common feature of these reactions is an interaction of peroxide with compounds which may serve as electron donors.

Study of the chemiluminescence process from the point of view of intra- and intermolecular electron transitions initially was used for explaining the mechanism of luminescence accompanying reverse photochemical reactions [34, 36] and during recombination reactions of anion radicals with strong oxidizers in model systems [141, 142]. This approach may also be fruitful in the study of other chemiluminescence reactions in chemical and biological systems.

Ultraweak Luminescence Accompanying Photochemical Reactions in Proteins

Strictly speaking, chemiluminescence is a process fully the reverse of a photochemical reaction only in one of the cases considered above -- in the recombination luminescence of frozen solutions of tyrosine, tryptophan and proteins irradiated by ultraviolet light at 77°K. In other cases luminescence did /70 not develop during reverse photochemical processes but during subsequent dark biochemical reactions of an active photoproduct (such as of an ion radical of chlorophyll) with molecules of the ambient medium. How these active products are formed -- during photochemical or during biochemical reactions -- no longer is of importance for the chemiluminescence mechanism as such.

What has been said above applies fully to the luminescence of protein films and solutions. The phenomenon of persistent afterglow of protein powders was discovered by F. F. Litvin and the author in 1959 [32, 33]. The measured afterglow spectra, which resemble the protein phosphorescence spectra [30], were not, however, given in our first studies. In 1961-1962 S. V. Konev and M. A. Katibnikov made a detailed study of the afterglow of films and solutions of a number of pro-

teins after ultraviolet irradiation [73, 80]. Study of the kinetics of attenuation [80] indicated that the process is not phosphoresence, but has a more complex nature. The excitation spectra of the protein afterglow had maxima at 220 and 280 mμ, which is characteristic for tryptophan absorption; the afterglow spectra of films had a maximum at 440 mμ [30, 73], which is close to the phosphoresence maximum of protein and tryptophan. All this indicates the central role of aromatic amino acids in this process. This also is indicated by the data obtained by D. I. Roshchupkin in our laboratory; these data show that solutions of pure tryptophan at room temperature have an afterglow.

Important observations relating to the mechanism of afterglow of proteins were made by I. I. Sapezhinskiy and associates [107-111]: these authors demonstrated that the afterglow of proteins in a solution increases sharply in the presence of oxygen [107]. The admission of oxygen into a vessel with a pre-irradiated solution of protein led to an intensification of the luminescence. At the same time, addition of cysteine and other antioxidants decreased the duration of luminescence attenuation and decreased its intensity [107, 111]. Afterglow also was quenched by such compounds as ionol and α-napthol -- well-known inhibitors of the peroxide radicals ROO·. These data made it possible to represent the protein afterglow mechanism in the following form [109, 111].

1. Formation of $\overset{\bullet}{R}_0$ radicals and their entry into solution

$$\overset{\bullet}{R}_0 \rightarrow \overset{\bullet}{R}$$
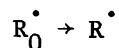
2. Recombination of radicals and luminescence

$$\overset{\bullet}{R} + \overset{\bullet}{R} \rightarrow h\nu_1$$
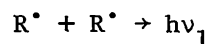
3. Formation of peroxide radicals in the presence of oxygen

$$\overset{\bullet}{R} + O_2 \rightarrow R\overset{\bullet}{O}_2$$
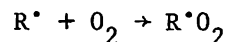
4. Recombination of peroxide radicals and luminescence

$$R\overset{\bullet}{O}_2 + R\overset{\bullet}{O}_2 \rightarrow h\nu_2$$
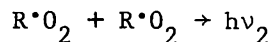
5. Interaction of a peroxide radical with an inhibitor, not accompanied by luminescence

$$R\overset{\bullet}{O}_2 + InH \rightarrow In\overset{\bullet}{} + ROOH$$

50
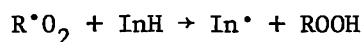
This model is an application of the Vasil'yev model to the afterglow of proteins and possibly in this case is in need of refinement, but we feel that in light of the experiments mentioned above the formation of peroxide radicals in the system is extremely probable.

As is not surprising, luminescence was observed in the experiments of Stauff and his associates after the irradiation of proteins by visible light: the maximum in the effect spectrum fell in the 400-mμ region. This afterglow was associated with the presence of sulfhydryl groups in protein. The oxidation of -SH and S-S groups of performic acid or their reduction and carboxymethylation resulted in the disappearance of afterglow. The same effect was exerted by n-chlormercuribenzoate, which blocks the -SH groups [196].

It is interesting to compare luminescence after the irradiation of proteins and under the influence of chemical agents. The addition of hydrogen peroxide to protein resulted in luminescence [109, 196]. Luminescence also was observed after the addition of glacial acetic acid or 8 M urea to protein in the presence of oxygen of the air [108]. This luminescence was attenuated in a vacuum and with the addition of inhibitors of free radical reactions. The luminescence maximum fell at 460-480 mμ [108]. Apparently there is much in common <u>/72</u> between prolonged afterglow and chemiluminescence in protein solutions (with the addition of H_2O_2). In particular, in both cases there was an activating effect of some dyes, especially eosine [110, 196]. This influence of eosine was specific for processes in which a protein participated; the decomposition of hydrogen peroxide in the presence of Fe^{++} and EDTA was accompanied by luminescence; the addition of eosine had no effect on its intensity [196].

Stauff assumes that the influence of eosine may be related to the migration of energy from aromatic amino acids (probably tryptophan) to a dye. The excited (possibly triplet) state of tryptophan arises as a result of some reactions in the protein molecule in which the sulfhydryl groups participate, on the one hand, and peroxide radicals of water or protein, on the other hand.

In these processes an important role also is played by the structure of the protein molecule: the addition of 8 M urea, 5 M guanidine sulfate and 0.1 M sodium dodecylsulfate led to the disappearance of protein in the experiments of Stauff and Wolf; it goes without saying that there was a simultaneous change in the optical rotation of the solution [196].

It should be added that I. I. Sapezhinskiy and his associates expressed doubt as to the identity of the luminescence mechanisms for proteins after ultraviolet irradiation and with addition of hydrogen peroxide on the basis that in the case of ultraviolet irradiation not more than 10^{-6} M hydrogen peroxide is formed, whereas for chemiluminescence in the system H_2O_2 + protein the peroxide concentration should be not less than 10^{-3} M [109]. Apparently it is not the hydrogen peroxide itself, but the peroxide radicals in a molecule of protein, forming either during the illumination of the solution in the presence of oxygen or under the influence of H_2O_2 in the darkness, which plays the necessary role in the reactions of prolonged afterglow and chemiluminescence of proteins.
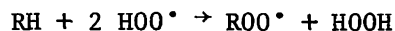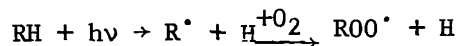
In summarizing the above, it is possible to conceive of a luminescence mechanism for protein solutions in the form of a hypothetical model.

1. Formation of peroxide radicals:

under ultraviolet irradiation:

$$RH + h\nu \to R^{\cdot} + H \xrightarrow{+O_2} ROO^{\cdot} + H$$

under the influence of peroxide radicals of water:

$$RH + 2\ HOO^{\cdot} \to ROO^{\cdot} + HOOH$$

or

$$RH + HOO^{\cdot} \to R^{\cdot} + HOOH$$

$$R^{\cdot} + O_2 \to ROO^{\cdot}$$
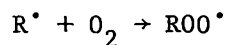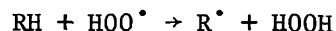
It is extremely probable that RH is an aromatic amino acid: tyrosine or tryptophan. The fact that under the influence of ultraviolet radiation, tyrosine and tryptophan are photoionized with subsequent detachment of a proton (formation of $R^{\cdot}$) has been demonstrated by a whole series of methods [3, 4, 30, 31, 42, 43, 104, 105, 162, 209, 210].

2. Decomposition of peroxide radicals with the formation of an excited (possibly triplet) state of aromatic amino acids:

$$ROO^{\cdot} + AH \to RH^{\nu}.$$

It is not impossible that the role of the AH component in this process may be played by the remnants of cysteine or cystine. The probability of an intramolecular interaction of $ROO^{\cdot}$ and SH groups in protein is dependent on the conformation of the macromolecule, which exerts an influence on the afterglow intensity of the proteins [196]. Thermal denaturation intensified the chemiluminescence of the serum albumin under the influence of H_2O_2 by four to five times [109].

3. Emission of a photon:

$$RH^{\nu} \to RH + h\nu_{phos}.$$
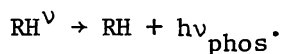
The probability of formation of a triplet molecule of amino acid (RH^{ν}) is greater than the probability of formation of a molecule in a singlet excited state and therefore the chemiluminescence spectra and prolonged afterglow in protein solutions and films should be close to the phosphorescence spectrum, which, as already mentioned, is actually observed.

4. At room temperature the quantum yield of a radiative transition from

the triplet state is very low*; therefore, the intensity of the luminescence increases sharply with the addition of an energy acceptor -- eosine [110, 196]:

$$RH^{\nu} + A \rightarrow RH + A^*$$

$$A^* \rightarrow A + h\nu,$$

where $h\nu$ is an eosine fluorescence quantum.

In conclusion, it should be noted that the luminescence associated with the recombination of peroxide radicals in a macromolecule apparently also was observed in experiments with nucleic acids. This is indicated by the study results on luminescence accompanying the heating of DNA solutions irradiated by x-rays [130] in which, as is well known, there are peroxides of purine bases [158, 188].

Molecules Emitting Quanta of Ultraweak Luminescence During Biochemical Reactions

Study of the activation of protein afterglow by luminescent compounds during aromatic hydrocarbon reactions reveals that the reactions supplying energy for the formation of an excited state and the processes of photon emission may be associated with different molecules. It would appear that the problem of which molecules emit a quantum can be solved in the final analysis on the basis of the luminescence spectra.

The precise measurement of the spectra of ultraweak luminescence in biological systems has not yet been done; an approximate evaluation of the spectrum, using light filters, has been accomplished repeatedly (the method was described in [16]). In particular, Table 6 shows the spectral region and the approximate position of the luminescence spectrum for the case of enzymatic decomposition of hydrogen peroxide. It is easy to see that the luminescence was characterized by the presence of two maxima: about 450-460 and about 510 mμ. The phosphorescence of protein and tryptophan [33] and the spectrum of protein after- /75 glow at room temperature [30, 32, 33, 73] lie approximately in this region. However, on the other hand, in this same region there is a strong luminescence accompanying the interaction of peroxides with a number of other compounds, which Stauff and Schmidkunz [194] attributed to luminescence from van der Waals oxygen complexes (maxima at 480, 535, 580 or 635 mμ) (see Table 3).

Thus, judging from the luminescence spectra, both tryptophan and oxygen can be "emitters" of a chemiluminescence quantum accompanying the effect of

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*I. I. Sapezhinskiy, Yu. V. Silayev and A. V. Kutsenova, on the basis of a quantitative study of the effect of sensitizing luminescence with eosine, used the method described in [12, 20, 21] for determining the quantum yield of luminescence of an energy donor; this value was $8.6 \cdot 10^{-4}$ [110]: this probably is the yield of tryptophan phosphorescence in protein at room temperature.

Reagents added to 1.5% solution of H_2O_2	Luminescence intensity, pulses/minute	Luminescence region, mμ	Approximate position of maxima
Solution of crystalline catalase	135	440–530	460, 510
Solution of crystalline peroxidase of horse-radish + tyrosine (1 mg/ml)	400	440–530	460–490
Suspension of yeast cells destroyed by acetone	147	420–530	450, 510
Rat liver homogenate, 0.2% solution of	87	450–530	460, 500–510
rabbit blood	645	440–530	470–480, 510

H_2O_2 on systems containing protein.

Under the influence of hydrogen peroxide on rat liver homogenates [100] it was observed that there also was a red luminescence characteristic of oxygen luminescence [133, 171]. Similar results were obtained during the oxidation of lipids, where both green (510–530 mμ) and red (> 640 mμ) components of luminescence are observed [117].
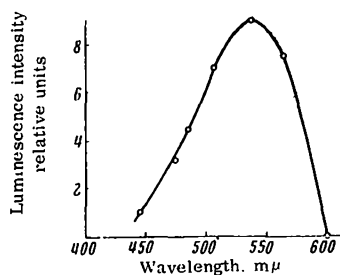
Figure 15. Luminescence Spectrum of a Mitochondrial Suspension Evaluated using Light Filters [41]. Composition of Incubation Medium Same as in Caption to Figure 5.

The luminescence spectrum of a mitochondrial suspension, incubated in a saline medium with respiration substrates and ATP in the presence of oxygen, and evaluated using light filters, is shown in Figure 15. In this case the luminescence maximum fell in the region 540 mμ and was close to the fluorescence maximum of flavins (530 mμ) and the maximum of chemiluminescence of riboflavin in the presence of hydrogen peroxide (650 mμ [200]). It therefore may be concluded that in this system there may be radiation not only of a $(O_2)_2$ or aldehyde molecule, but flavin nucleotides as well [41]. The latter is all the more probable in that the formation of unsaturated fatty acids activated by coenzyme A, and therefore lipid peroxides, as shown by the model following Figure 7, can occur in reaction 2, associated with the participation of flavoproteins.

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The examples given here clearly show that the study of spectra of ultra-weak luminescence even in model systems is only beginning, and for the time being do not make it possible to state with assurance exactly what molecules are in an excited state during bioluminescence. Therefore, to ascribe the radiation of intact cells to any definite biochemical compound, such as proteins or nucleic acids, on the basis of approximate evaluations of the radiation spectra [77], appears to the author to be premature.

Molecular Mechanisms of Bioluminescence

The ultraweak luminescence from animal tissues, caused, as we now know, to a considerable degree by mitochondrial oxidative phosphorylation reactions, is not the only form of chemiluminescence from biological matter. Among such phenomena is the rather strong luminescence* of some bacteria, fungi, fireflies and other animals, long known under the term bioluminescence. It is of undoubted interest to compare the "exotic" mechanism of bioluminescence with the universal mechanism of ultraweak luminescence in mitochondria.

Investigators of bioluminescence long have noted that this rather rare phenomenon is observed in animals belonging to highly different systematic groups and that the mechanisms of luminescence in different species differ greatly [181]. It is the mechanism of luminescence in the firefly which has been studied most [180, 181, 186] (see diagram):

$$
\begin{array}{c}
\text{PP} \\
\downarrow \leftarrow \\
\text{E}-\text{ATP}+\text{L}=\text{O} \\
\downarrow \leftarrow \text{LH}_2 \\
\text{E}+\text{LH}_2+\text{ATP} \rightleftarrows \text{E}-\text{LH}_2-\text{AMP}+\text{PP} \\
\downarrow \leftarrow \text{O}_2 \\
\left[\begin{array}{c} \text{E}-\text{L}-\text{AMP} \\ \parallel \\ \text{O} \end{array} \right]^* +\text{H}_2\text{O} \\
\downarrow \\
\text{E}-\text{L}-\text{AMP} \\
\parallel \qquad + \text{LIGHT} \\
\text{O}
\end{array}
$$
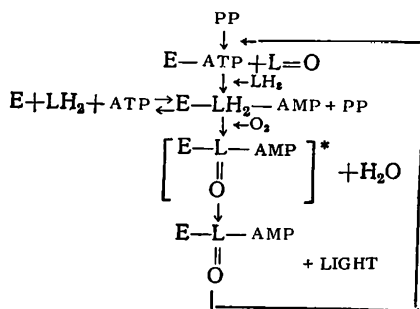
In this diagram LH_2 is luciferin, L = 0 is /78 oxyluciferin, E is luciferase, ATP is adenosine triphosphate, and PP is pyrophosphate. Light emission occurs as a result of the oxidation of the enzyme-substrate complex $E-LH_2-AMP$ by molecular oxygen.

Two aspects of the bioluminescence of the firefly are of the greatest interest to us. First, that the oxidation of luciferin is not simply dehydration but also the subsequent joining of oxygen, so to speak, "over-oxidation" with the formation of a keto group [182]:
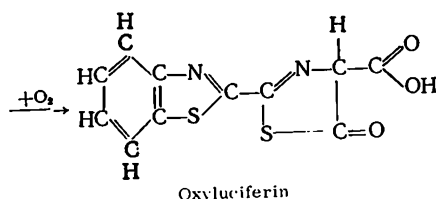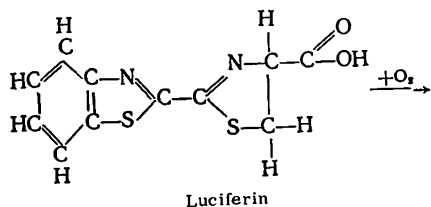
Luciferin

Second, the oxidation of luciferin not only requires interaction with luciferase, but also the formation of a complex with ATP.

Oxyluciferin

Although the luminescence of the firefly is caused by a specialized mechanism which performs a definite biological function and is controlled by the nervous system, its mechanism has a number of characteristics in common with the ultraweak luminescence from rat liver mitochondria, which scarcely can be deemed to perform any specialized biological function:

1) both types of luminescence fall in a close region of the spectrum, which may indicate a closeness of the "luciferins" in both

*The quantum yield of bioluminescence in the firefly is of the order of unity [63]; in Cypridina hilgendorfii it is 0.28 [170].

cases;

2) both processes undoubtedly have an enzymatic nature;

3) in both cases oxygen and adenosine triphosphate are necessary.

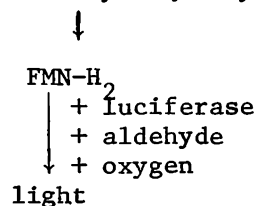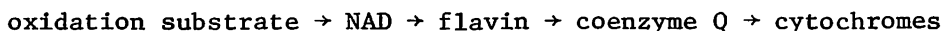Undoubtedly there also is a certain similarity between the ultraweak luminescence mechanism of mitochondria and the bioluminescence of bacteria because both phenomena are closely associated with the electron transport system. The bioluminescence of microorganisms in all probability includes the abundant energy formation of the reduced flavin mononucleotide oxidation product and transpires in conformance with the following scheme [92]:

$$\text{oxidation substrate} \to \text{NAD} \to \text{flavin} \to \text{coenzyme Q} \to \text{cytochromes}$$

$$\downarrow$$

$$\begin{array}{l} \text{FMN-H}_2 \\ \quad\mid\quad + \text{ luciferase} \\ \quad\mid\quad + \text{ aldehyde} \\ \quad\downarrow\quad + \text{ oxygen} \\ \text{light} \end{array}$$

Judging from the fact that in the model system $FMN-H_2$ + luciferase + aldehyde the luminescence maximum does not lie at 530 mμ (flavin fluorescence), but at 490 mμ , it is not flavin but certain groups of luciferase which are responsible for the bioluminescence. Hastings, Gibson and their associates proposed a model for explaining the mechanism of the phenomenon:
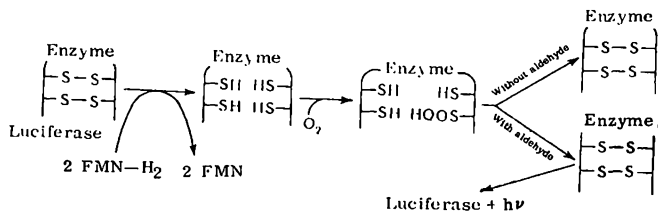
In the range from -10 to 0°C, when nonradiative deactivation of the excited state of luciferase is absent, bioluminescence with and without aldehyde had the same quantum yield [166].

As shown by the model presented here, the molecular mechanism for bacterial bioluminescence is surprisingly similar to the mechanism of protein ultraweak chemiluminescence in the presence of hydrogen peroxide. The similarity is increased by the fact that luciferase, like ordinary proteins, has afterglow, and its maximum excita- tion spectrum (280 and 400 mμ) [166] coincides with the maximum of other proteins [73, 196].

Thus, the bacterial bioluminescence, like mitochondrial ultraweak luminescence, is associated with the electron transport system, and the capacity of luciferase to participate in chemiluminescence reactions to a certain degree is characteristic of any protein.

"Purely protein" bioluminescence is characteristic of certain other organisms. For example, it was possible to obtain "crystalline" protein particles

from Gonyaulax polyhedra 0.1-0.6 μ in diameter, having a capacity for lumines-cence in the presence of oxygen. Light emission begins with a simple decrease of the pH of the solution containing "crystallines" [151, 152].

Without discussing in detail the extremely specialized mechanism of Cy-pridina bioluminescence, where luciferin also apparently has a flavin nature, we will consider the luminescence of Balanoglossus. In this case the lumines-cence has a completely different molecular mechanism and luciferin, luciferase and hydrogen peroxide are observed in the system. Luciferase is inhibited by cyanide and can be replaced by a crystalline peroxidase of horseradish; it therefore may be concluded that it has a peroxidase nature [147]. As we already have stated, the decomposition of hydrogen peroxide by crystalline peroxidase in itself also can cause weak luminescence.

A more detailed familiarization with bioluminescence can be obtained by consulting the reviews in [63, 148, 181].

Table 7 gives some examples of luminescent organisms.

The most surprising conclusion which can be drawn from a comparison of bioluminescence and ultraweak luminescence in mitochondria is that the latter has features in common with all the mentioned forms of bioluminescence, despite the fact that they differ appreciably from one another. For example, mito-chonrial luminescence requires ATP, similar to the bioluminescence of the fire-fly, and is intensified by a substrate of cellular oxidation, as bacterial bioluminescence, and finally, is suppressed by cyanide, like the biolumines-cence of Balanglossus. We feel that this fact is not random. It may indicate that all bioluminescence may be the result of the specialized development of /81 some part of a single initial mechanism, which could be the mechanism of elec-tron transport and conjugate reactions with the participation of proteins and lipids.

TABLE 7. SOME EXAMPLES OF LUMINESCENT ORGANISMS [92]

Organisms	Luminescence-prompt-ing reaction	Irradiation maximum, mμ
Luminescent bacteria	$FMH-H_2 + RC \overset{O}{\underset{H}{}} + O_2 + E$	495
Fireflies	$LH_2 + ATP + Mg + O_2 + E$	562
Cypridina (crusta-ceans)	$LH_2 + O_2 + E$	460
Odontosyllis (poly-chaetous worms)	$LH_2 + O_2 + E$	510
Pholas dactylus (elas-mobranchii (mollusks))	$NAD-H + FMH + O_2 + E$	480

TABLE 7. (Continued)

Organisms	Luminescence-prompting reaction	Irradiation maximum, mμ
Omphali flavida (fungi)	$NAD-H + X + O_2 + E$	530
Renilla reniformis (sea worms)	$LH_2 + AMP + O_2 + E$	blue region of spectrum
Gonyaulax polyhedra (protozoans)	$LH_2 + E + O_2$	470
Apogon (fishes)	$LH_2 + E + O_2$	460

Origin of Bioluminescence and its Relationship to Ultraweak Luminescence

McElroy and Seliger postulated that the oxidation reaction involving strong reducing agents by molecular oxygen, catalyzed by a special enzyme -- luciferase -- and accompanied by luminescence, already developed in those remote times when there was little oxygen on the earth and all organisms were anaerobic. Oxygen was poisonous for these organisms and they bound it, using a reaction whose basis may be considered present-day bioluminescence. This reaction appeared earlier than the present-day electron transport system and in itself did not lead to the storing of energy. In most organisms it was suppressed with the development of present-day oxidative phosphorylation, but it was retained in /82 some bacteria and in a number of organisms this reaction was used [92]. A rational nucleus of the McElroy-Seliger hypothesis is the idea that initially the bioluminescence reaction did not have adaptive importance: luminescence simply could not arise during the energetic oxidation of a strong reducer by molecular oxygen. At the same time, many factors remain unclear.

First, as pointed out by R. I. Chumakova, it is improbable that the bioluminescence mechanism was developed for rendering molecular oxygen harmless. In this case bioluminescence would be characteristic only of obligate anaerobes, whereas existing species of luminescent bacteria are facultative anaerobes [126]. Probably it was not the elimination of oxygen, but active enzymatic deactivation of extremely poisonous spontaneous oxidation products such as peroxides [75, 85, 89], which could be the starting material, accompanied by luminescence.

That part of the McElroy-Seliger hypothesis which explains the variety of bioluminescence mechanisms in the animal world also causes serious objection. In essence, the authors attribute it to the variety of reduction products which ancient organisms could use for eliminating oxygen. We feel that this great variety of independent evolutionary lines was quite improbable.

As we have already mentioned, in the liver mitochondria the luminescence

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mechanism has features in common with the bioluminescence of different organisms, and we feel it is extremely probable that the bioluminescence of all these organisms was created on the basis of the secondary development of side chains of the unified present-day electron transport system. In the course of evolution the energetically disadvantageous expenditure of chain chemical energy of reduced compounds on the direct interaction with oxygen was suppressed and the light nature of this biochemical energy was hidden from the eyes of researchers. The abnormally high luminescence activation energy in homogenates (55-57 Cal/mole) indicates that in the cell there are very high energy barriers preventing autoxidation and chemiluminescence; these are supplemented by structural bar- /83 riers. Only individual molecules are capable of penetrating through these barriers and the luminescence arising in this process can be detected only by use of highly sensitive apparatus. Catalase, peroxidase and possibly some luciferases destroy the randomly forming poisonous peroxides. Hundreds of millions of years had to pass in order for the energy in the substrates and reduced coenzymes, which is of a "light" nature, to begin to be expended in small quantities and stored in the "macroerg" pyrophosphate bonds at ATP.

<u>Biological Role of Processes Associated with</u>
<u>Ultraweak Luminescence</u>

It is necessary to distinguish the problem of the biological role of ultraweak luminescences in themselves and the role of processes which are accompanied by luminescence.

In reality, our knowledge concerning the importance of weak luminescence for the vital processes of cells and tissues has changed little since the work of A. G. Gurvich [52]. Since mitogenic rays were discovered with a biological detector, it is obvious that ultraweak ultraviolet radiation may exert a definite influence, at least on the cells of this detector.

According to the ideas of A. G. Gurvich, mitogenic radiation not only exists, but is necessary for the onset of normal mitosis in the cell. After the addition of a mitogenic radiation quencher, obtained from the blood of cancer patients, to yeast cultures, there was virtually no budding of yeasts during the first hour [55]. This same quencher (which is absent in the blood of healthy persons) delayed the growth of cells in a tissue culture, oogenesis in the guinea pig and mitosis in the cornea of the eye. The irradiation of cells by an external source of mitogenic rays restored normal cell division. On the basis of this type of experiment, A. G. Gurvich concluded that "the event of cell division is determined in its entire course by the mitogenesis of the cells" ([54], p. 13). Although this concept is essentially the basis of Gurvich's teaching, only one part of it -- the <u>existence</u> of radiation -- acquired reknown /84 and was subjected to criticism, whereas the other part -- the <u>effect</u> of ultraweak radiation on cells -- was overlooked by biologists. Unfortunately, neither Gurvich himself nor subsequent researchers gave sufficient attention to the systematic study of the effect of weak ultraviolet radiation doses from an outside source on cells, and very few data have been accumulated in this field [38, 86, 103]. Therefore, the problem of the biological importance of ultraweak luminescence in the ultraviolet region of the spectrum remains unclear.

Ultraweak radiations transfer an insignificant part of the vital energy of the cell. Even in such brightly luminescent organisms as luminescent bacteria, only one millionth of the oxidation energy of glucose is released in the form of bioluminescence [49]. In ordinary cells an energy of several orders of magnitude less is released in the form of ultraweak luminescence. This means that in the energetics of the living organism the formation of excited molecules plays no role [37]. However, the luminescence indicates that there are important events transpiring in molecules and in the cell. As we have seen, luminescence accompanies the formation of peroxides and radical recombination; these products, even in insignificant concentrations, exert a strong biological effect. For example, fatty acid peroxides are very strong radiomimetics [89], apparently as a result of their effect on the chromosome structure of the cell [132]. A similar effect is exerted by aromatic amino acid radicals [75] and the reaction products of sulfhydryl compounds and hydrogen peroxide [8]; such peroxides are possibly formed also during the oxidation of protein sulfhydryl groups by molecular oxygen [128]. In other words, the presence of luminescence indicates that something is not in good order in the investigated biological system. This also applies to more complex systems, e.g., mitochrondrial cell suspensions; the radiation in them is associated with the disruption of normal cell metabolism and its nature resembles that of the necrobiotic rays described by Lepeshkin. Disruption of the cell membrane structure by a temperature decrease led to a burst of luminescence from plant stems during experiments carried out by A. Sh. Agaverdiyev and B. N. Tarusov [2].

However, this is only one aspect of the matter. In the study of mito-   /85
chondria it becomes obvious that ultraweak luminescence is assoicated, on the one hand, with normal metabolism (oxidative phosphorylation), and on the other hand -- with the disruption of this metabolism (accumulation of peroxides and release of oxidation and phosphorylation). This apparent contradiction can be attributed to the fact that the formation of lipid peroxides and luminescence reflects the process of mitochondrial, swelling, which plays an exceptionally important role in cell physiology and pathology. Under normal conditions the mitochondrial membrane participates in regulating the rate and efficiency of oxidative phosphorylation [116] and also the rate of glycolysis in the protoplasm and nucleus (due to the release of coenzymes and lipoprotein in the cell [95, 96]. Mitochondria probably to some degree also regulate the functioning of the genetic apparatus of the cell; in particular, it has been reported that mitochondria contain a factor that slows down the formation of antibodies [10]. Ultraweak luminescence does not simply reflect the accumulation of lipid peroxides in membranes; it is a direct result of a reaction which is extremely important for changing mitochondrial membrane permeability: interactions of the peroxide radical either with a molecule of intracellular antioxidant or with the sulfhydryl group of protein, such as actomyosin, which is responsible for the contraction of mitochondria (see p. 31-34).

Thus, ultraweak luminescence to a considerable degree is caused by processes in the cell membrane. The facts now accumulated also indicate that ultraweak radiation may exert an effect on the cell and this apparently occurs through an effect on the cell membrane. For example, in the experiments of A. G. Gurvich, ultraweak ultraviolet rays exerted the most effective influence on yeast cells ready for budding; it is possible that the radiation did not

cause the mitosis (that is, was not mitogenic) but only activated gemmation, associated with breaking of a part of the membrane. The activating effect of weak ultraviolet radiation on phage particles forming in the bacterial cell is known (for example, see [38]); this process also is associated with changes in the bacterial cell membrane. We already have mentioned the influence of mito- /86 chondrial membrane permeability on glycolysis. S. V. Konev and T. I. Lyskova have demonstrated that very weak ultraviolet radiation sharply activates glycolysis in a yeast culture (reference in [78]). At the same time it accelerated budding in a synchronous culture [81].

The literature contains repeated discussions of the problem of using ultra-weak luminescence for recording the pathological states of a cell, especially for cancer diagnosis. As is well known, cancer cells are characterized by their aggressiveness, extremely high lipid consumption [187] and capacity for aerobic glycolysis, which is due to the high and uncontrolled mitochondrial membrane permeability [95]. The strong mitogenic radiation from cancer cells and the quencher of this radiation they release into the blood possibly somehow are related to these properties [55]. Interesting data also state that the lipid fraction of cancer cells gives considerably stronger luminescence after addition to oxidized oleic acid than a fraction from normal tissue [67]. A further study of these problems undoubtedly will make it possible to clarify what aspects of cancer cell metabolism can be studied by registering ultraweak luminescence and in what cases this method can be used for the diagnosis of cancerous conditions.

The energy of any biochemical reaction is solar energy stored during photosynthesis and gradually expended in the course of other biochemical processes. However, whether this energy within the body could be released again directly in the form of light quanta remained unknown for a long time. A basic solution of this problem became possible only after the development of sensitive and stable photon counters, which were based on photomultipliers with a low dark current, and especially photomultipliers cooled by liquid nitrogen. Several trends in research have been noted. First, a study is being made of an increasing number of biological objects having luminescence. Second, a detailed phenomenological investigation of the phenomenon is being made: study of the spectral region of radiation, influence of pH, temperature, oxygen, etc. Third, increasing attention is being given to a clarification of the biochemical reaction mechanism, which leads to the accumulation of products responsible for the luminescence (for example, peroxide radicals). Finally, attempts are being made to understand the molecular mechanisms of quantum generation in the final stage of chemiluminescence in chemical and biological systems. The increasing interest shown by researchers concerning the problem of ultraweak luminescence makes it possible to surmise that this branch of biophysics will develop further in the near future.

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